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14. ABSTRACT
Impaired social behavior is a treatment-resistant core symptom of autism that also manifests in other psychiatric disorders. Selective serotonin reuptake inhibitors (SSRIs) such as Prozac (fluoxetine) are capable of enhancing sociability in some patient sub-populations, but their efficacy is greatly diminished if 5-HT transporter (SERT) function is compromised. For this reason, our goal was to characterize effects of blocking ancillary transporters of 5-HT instead of SERT. These auxiliary transporters, known as 'uptake 2', include organic cation (OCT) and plasma membrane monoamine transporters (PMAT) which exhibit lower affinity but greater capacity than SERT to remove 5-HT from extracellular fluid. Through synaptosomal uptake and radioligand binding, we found the pseudoisocyanine decinium-22 (D-22) blocks 5-HT uptake (Km=92±12 nM) but has negligible affinity for the SERT (Ki > 3000 nM). D-22 (1 mg/kg, i.p.) is cleared from mouse serum with a half-life ≈30 min, with some variability among strains. We used inbred strains BTBR, 129S1/SvIMJ and SERT knock-out (-/-) mice, exhibiting impaired social behavior relative to wild-types (+/+) on C57BL/6 background or DBA1, to examine acute and chronic effects of D-22 on sociability. Social sniffing and dwelling near strangers increased in BTBR and SERT -/- mice on D-22 (0.01-0.1 mg/kg), relative to vehicle-controls. Two-week D-22 (0.01-0.001 mg/kg/d) administration also improved BTBR and SERT -/- mouse sociability. Thus, uptake 2 blockade may be an effective strategy to ameliorate social behavior impairments.

15. SUBJECT TERMS
autism, auxiliary transporters, corticosteroids, decynium-22, impulsivity, inbred mice, interaction preference, monoamine uptake blockade, organic cation transporters, pseudoisocyanine, repetitive behavior, serotonin, social behavior

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INTRODUCTION: Social interaction deficits are prominent core behavioral symptoms of autism that are particularly challenging to treat. The selective serotonin (5-HT) reuptake inhibitor (SSRI) fluoxetine is one of a few effective frontline pharmaceutical interventions for autism symptoms. Restrictive-repetitive behaviors in autism are effectively diminished by the atypical antipsychotic risperidone or SSRI fluoxetine in clinical trials (Soorya et al., 2008; West et al., 2009a). However, social behavior is enhanced by SSRI treatment in only small populations of autistic patients (Henry et al., 2006; 2009; West et al., 2009b, McPheeters et al., 2011). This relatively poor therapeutic outcome brings into question the utility of SSRIs for treating impaired social behavior in autism (Williams et al., 2013). SSRIs inhibit 5-HT uptake by blocking the high-affinity 5-HT transporter (SERT), the primary regulator of extracellular 5-HT in brain. However, low-affinity, high-capacity auxiliary transporters of 5-HT, collectively referred to as 'uptake 2', include organic cation and plasma membrane monoamine transporters (OCTs and PMAT). These auxiliary transporters also play a significant role in regulating 5-HT transmission (Baganz et al., 2008; Daws, 2009; Duan & Wang, 2010; Hill et al., 2011; Daws et al., 2013). Recent studies show the activity of these ancillary 5-HT transporters can dampen the therapeutic effects of SSRIs in depression-related mouse behavior tests (Baganz et al., 2008; Daws 2009; Daws et al., 2013; Horton et al., 2013). This raises the possibility that the poor efficacy of SSRIs to improve impaired social behaviors in autism might also be due to their activity. Given this, we hypothesized that blockade of these transporters (OCT3 and/or PMAT) might effectively ameliorate impaired social behavior. The pseudoisocyanine decynium-22 (D-22) is an effective blocker of uptake 2 transporters, so in this study we examined its affinity for the SERT versus auxiliary "uptake 2" transporters, and tested its pharmacokinetic and behavioral properties in socially-deficient BTBR, 129S1/SvImJ and SERT knock-out mice as compared to in more sociable control strains.

BODY: Post-Award Revised Statement of Work: The aims of this three-year study were to: 1) Establish the pharmacokinetic properties of D-22 administered acutely in mice through measurement of the parent compound and its metabolites in serum and brain, and to identify doses and exposure durations eliciting maximal social behavior effects, but not sedation. 2) Determine the ability of D-22 to reduce hippocampal serotonin (5-HT) uptake in vitro and in vivo in socially impaired BTBR and 129S1/SvImJ mice, as compared to relatively gregarious C57BL/6 and DBA/1J strains; and 3) Establish dose-response relationships for D-22 administration, alone and in combination with risperidone or fluoxetine, as compared to fluoxetine and risperidone treatments for improvement of social interaction with confined stranger mice, and advancing and retreating behavior in the tube test for social dominance, and on restrictive repetitive behavior in these strains. The PI, Co-I, and all Collaborating Consultants are at the University of Texas Health Science Center Facility, and all experiments will be performed on-site, so there were no sub-awards for the proposed project.

Personnel: Principal investigator: Dr. Georgianna Gould, Initiating PI, Department of Physiology

Co-investigator: Dr. Lynette Daws, Co-PI, Department of Physiology

Collaborator: Dr. Martin Javors, Consultant-collaborator, Department of Psychiatry

Collaborator: Dr. Wouter Koek, Consultant-collaborator, Department of Psychiatry

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Task 1. Seek necessary regulatory reviews and approvals for proposed research:

1a. Certificate of Environmental Compliance for UTHSCSA (upon award notification): provided at study initiation

1b. Complete Principle Investigator Safety Program Assurance form (upon award notification): provided at study initiation

1c. Complete IACUC and ACURO "Research Involving Animals" protocols (upon award notification): Approval notices from IACUC and ACURO were obtained, and annual progress updates were submitted, approved and provided to protocol review specialist Joseph J. Kallhoff, RLATG, ILAM USAMRMC Animal Care & Use Review Office Fort Detrick, Maryland each year of this three year study.

1d. Obtain colony founders from supplier and/or set up breeding pairs to assure sufficient mice of each strain or genotype for all studies (month 1 and throughout study) (112 mice, 36-38 mice per year): This was accomplished in each year of the study, sufficient numbers of all mouse strains were continuously available as needed.

Task 2. Pharmacokinetic and behavioral characterization of acute D-22 administration in mice:

2a. In BTBR and C57BL/6 mice, commence pharmacokinetic studies with D-22 (240 mice) In year 1, to determine if systemically injected decynium-22 (D-22) could cross the blood brain barrier, intraperitoneal (i.p.) injections of 10 mg/kg D-22 dissolved in 10% DMSO, 90% saline into 3 male C57BL/6 mice. Thirty minutes later mice were sacrificed, trunk blood was collected and serum isolated. Measurement of D-22 concentration by HPLC (Javors Lab) revealed roughly 50 pg/mg of D-22 was found in brain (Horton et al., 2013). This published HPLC procedure was used to perform analysis of D-22 levels in the brain and blood for subsequent analyses. Another small study was conducted in year 1 to determine D-22 clearance rate. In adult male BTBR mice D-22 (injected at 1 mg/kg i.p.) had a half-life 35 min, based on sacrifice points at 5, 20 or 50 min after injection (**Fig. 1**). This preliminary data guided subsequent modifications to the design of our pharmacokinetic studies, specifically the 4 hr 12 hr and 24 hour time-points were dropped in favor of more times under 1 hour, since D-22 was not likely to be detectable in serum by HPLC beyond 2 hours.

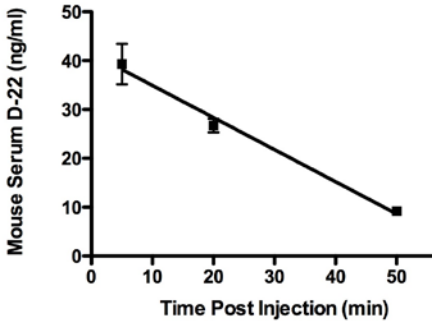

Fig. 1. Clearance of 1 mg/kg decynium 22 (D-22) from adult male BTBR mice. The half-time for clearance was approximately 35 min, N =3 mice/point.

i. Determine D-22 metabolism and baseline serum corticosterone levels (months 2-8)

a. Measurement of serum and brain D-22 levels by HPLC (months 6-12)

In a subsequent and larger pharmacokinetic analyses in year 1, 18 each BTBR, C57BL/6 and 129S1/SvImJ (129S) mice were injected with 10 mg/kg D-22 (dissolved in a 10% dimethylsulfoxide (DMSO), 90% saline solution) and sacrificed after i.p. injection at 15, 30, 45, 60 or 120 min, with 3 mice per time-point. Many of the mice at the 60 and 120 min time-points exhibited toxic effects, particularly those of the C57BL/6 strain, and were cold to the touch, although respiration was evident from movement of the ribcage and air movement at the mouth. The resultant clearance curve from blood is shown in Fig. 2. Due to high variability half-time estimates could not be resolved with linear regression for this concentration of D-22. However concentration and clearance of D-22 from brain tissue at this concentration should provide valuable information about occupancy at its key binding sites, including the OCT3. In the next round of experiments 18 BTBR and 18 129S mice were injected i.p. with D-22 at 1 mg/kg (in 10% DMSO, 90% saline vehicle) and were sacrificed at 0, 15, 30, 45, 60 or 120 min. In this group some of the locomotor activity was reduced in

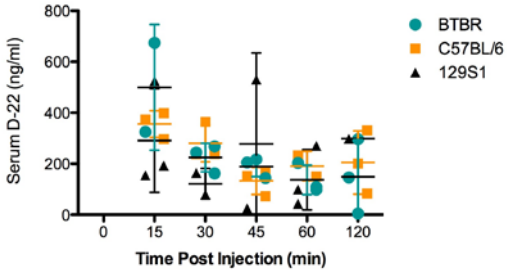

Fig. 2. Clearance of D-22 (10 mg/kg) from serum in three mouse strains. N= 3 per time point, bars represent mean and standard error.

the 129S mice at 60 min, but otherwise no adverse effects were noted. The serum and brain samples were collected and HPLC analysis was only very recently performed, and results are not yet available. There were insufficient numbers of C57BL/6 and DBA1 mice to utilize in this round of pharmacokinetic analysis of D-22. Due to limits of detection, it was deemed unlikely that HPLC analysis can be performed at doses below 0.1 mg/kg, so in year 2 and 3 of the study we transitioned to GC/MS.

b. Measurement of serum corticosterone levels by EIA (months 6-8)

Comparative analysis of serum corticosterone (CORT) levels between strains at baseline, and following social interaction tests was published in two manuscripts, the first one demonstrated that baseline CORT levels were higher in BTBR mice than in C57 or 129S1/SvImJ mice, and that social interaction testing itself raises serum CORT levels in both adults and adolescent mice that are 35 days old (Gould et al., 2014). The second manuscript demonstrated that in adult C57BL/6, BTBR and 129S1/SvImJ mice sociability testing increased serum CORT (Zhang et al., 2015). This is important, since previous studies in our laboratory and in others indicate that CORT can inhibit 5-HT uptake in vivo, most likely via uptake 2 transporters (Baganz et al., 2008; Hill et al., 2011). Baseline serum CORT levels of adult male BTBR and C57BL/6 mice were measured after behavior test using an ELISA kit, and we observed means of 109 ± 11 and 78 ± 6 ng/ml, respectively, they were significantly different (two-tailed $t = 2.42$, $df = 8$, $p < 0.05$). We collected serum samples from most mice used for behavioral tests and froze them for further measures of CORT levels, and have measured many using EIA kits beginning in March 2016, analysis of these samples is still underway.

ii. Perform behavioral dose-response tests in 30% of mice prior to HPLC (months 6-11)

We examined effects of D-22 on the social behavior of BTBR mice at a range of doses in comparison to diazepam (1 mg/kg), a known positive control to improve sociability in BTBR mice (Pobbe et al., 2011). D-22 and diazepam were dissolved in DMSO that was diluted to >10% of the saline vehicle administered to mice. Mice were injected (i.p.) with vehicle (saline + ≈10% DMSO) or D-22 (0.001 – 1 mg/kg), 30 min prior to arena acclimation. Mouse sociability tests were performed as in our prior studies (Gould et al., 2014, Zhang et al., 2015). As shown in Fig. 3, D-22 at doses ranging from 0.1–0.1 mg/kg (N=7-10) resulted in an increase in interaction preference that was evident in social sniffing. Further, there was no loss of social novelty preference typical of BTBR mice in the second stage of testing, while by contrast diazepam reduced the inherent social novelty preference of BTBR mice. In the third year of the project we performed additional studies with BTBR mice to increase some of the sample sizes for some of the doses with high variance. These newer data are currently being analyzed as part of a summer high school student's project.

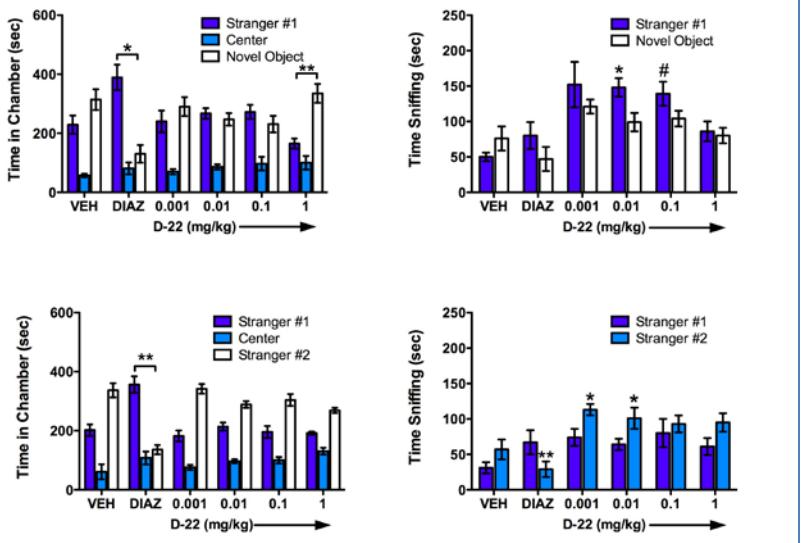

Fig. 3. D-22 Dose-Response in BTBR 3-Chamber Sociability Tests. In social interaction tests (top) only diazepine (DIAZ) increased dwelling by stranger mice (*p < 0.05), while 1 mg/kg of D-22 increased dwelling near novel objects. However, D-22 at 0.01 mg/kg (*p < 0.05) increased social sniffing, and tended to do so at 0.1 mg/kg (#p = 0.1). In social novelty tests (bottom) DIAZ significantly reduced preference for social novelty, both in dwelling and sniff time measures (**p < 0.05). In contrast, D-22 maintained the BTBR preference for social novelty [10], and increased

iii. Analyze data, manuscript and poster presentation to disseminate results (month 12)

The PI is currently preparing a manuscript to present this dose-response data demonstrating that D-22 promotes BTBR sociability acutely. Poster presentations were made at national meetings every year in order to disseminate results. These are listed under "reportable outcomes".

2b. For DBA and 129S mice perform pharmacokinetic and behavior analyses (240 mice)

In year two of the study, measurements of D-22 injected at a dose of 1 mg/kg were performed in serum from BTBR and C57 mice sacrificed at 0, 15, 30, 45, 60 and 120 min after injection, as Fig. 4 shows. Adult male BTBR mice were injected with D-22 at 1 mg/kg i.p. It had a half-life 35 min, based on post-injection sacrifice points at 5, 20 or 50 min. This data guided subsequent modifications to the design of our pharmacokinetic studies, specifically the 4 hr 12 hr and 24 hour time-points were dropped in favor of more times under 1 hour, since D-22 was not detectable in serum by HPLC beyond 2 hours. Due to initial struggles with detection limits for HPLC measurement of D-22 in brain samples, Dr. Javors' technical staff put much effort into optimizing the HPLC protocol than initially anticipated to obtain these results shown for BTBR and C57 mice. D-22 at 1 mg/kg was also injected into 129S and DBA1 mice, and tissue samples were collected for all of the 15 min to 2-hour time points.

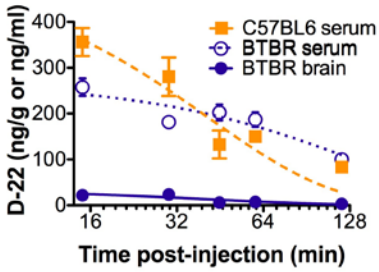

Fig. 4. D-22 clearance in adult mice. Half-life for D-22 (1 mg/kg) clearance from serum was ≈29 min in C57, ≈90 min in BTBR mice. D-22 was detectable in BTBR mouse brain at 15 min, with half-life ≈28 min.

i. Determine D-22 metabolism and baseline serum corticosterone levels (months 13-20)

a. Measurement of serum and brain D-22 levels by HPLC (months 18-24)

As an alternative approach Dr. Javors suggested for lower concentrations of D-22 found in brain samples was to measure D-22 by gas chromatography/mass spectroscopy (GC/MS) instead to achieve better sensitivity in detecting concentration differences closer to the behaviorally active dose range. We requested from IACUC an increase in mouse numbers to repeat the pharmacokinetic studies in all 4 mouse strains in anticipation of the GC/MS protocol coming on line. A new batch of (N= 1-3 per time point) 129S, DBA, C57BL/6 and BTBR mice were injected with 0.5 mg/kg of D-22 and brain and serum and blood samples were collected and frozen for subsequent analysis. GC/MS measurements of intraperitoneally-injected D-22 at a dose of 0.5 mg/kg was performed in 163 mice of strains BTBR, C57BL/6, 129S1/SvImJ and DBA1. The mice were sacrificed at 1, 15, 30, 45, 60, 75 and 100 min after injection. Whole brains were collected for measurement by GC/MS and serum by HPLC. The GC/MS approach was under development for most of the year, and now appears to be rendering accurate measures. Brain D-22 concentrations were as follows: 5 ± 1 pm/mg in C57BL/6 (N = 25), 2.5 ± 1 pm/mg tissue in DBA (N = 6), 6 ± 1 pm/mg tissue in 129S (N = 33), and 5 ± 2 pm/mg tissue in BTBR mice (N = 22). Since there is little variability across time-points in brain D-22 concentrations, it appears these injections reach sufficient concentrations in the brain to occupy either the target organic cation transporter (OCT), plasma membrane monoamine transporter (PMAT) binding sites within a minute of injection, and these concentrations are sustained throughout the timeframe of behavior testing. This finding confirms our prior published account that D-22 injected systemically at 10 mg/kg enters the brain (Horton et al., 2013). Corresponding serum concentrations have yet to be measured as one of the pumps on the GC/MS is in need of repair, the measures will resume some time before the end of September. While the GC/MS approach took a little longer to develop, we anticipate being able to complete all remaining serum D-22 measures in the next few months in preparation for a manuscript.

b. Measurement of serum corticosterone levels by EIA (months 18-20)

We continued to collect serum to measure corticosterone levels after behavior tests, but did not observe any effect of drug treatment in this measure. More assays were recently performed and their statistical analysis is underway, and additional EIA kits have been procured to analyze the remaining serum samples.

ii. Perform behavioral dose-response tests in 30% of mice prior to HPLC (months 18-24)

We have continued to test a range of doses (0.01 - 1 mg/kg) of D-22 in comparison to risperidone, fluoxetine or vehicle on social behavior in 129S mice. Two years ago the data from 129S mice were highly variable,as shown in **Fig. 5**. In 129S mice we observed that a change in diet from Harlan Teklad cereal based chow to a purified diet dramatically improved their social behavior (Zhang et al., 2015). We have conducted further tests with D-22, risperidone and fluoxetine treatments in order to increase the sample sizes and resolve any differences in response to drug treatments by this measure in 129S mice. The behavior tests were performed and video analysis is underway. Also in DBA mice we have performed more experiments examining the effects of D-22 at 0.1 mg/kg and combined D-22 and fluoxetine treatment effects in 8 mice per treatment group to add to the data reported in year 1 (**Fig. 6**),. These videos from DBA1 mice were only recently analyzed. Further studies were also performed in C57BL/6mice to clarify effects of D-22 at two doses. D-22 did not alter C57BL/6 sociability at 0.001 or 0.01 mg/kg. With these additional experiments completed, our group is poised to compose manuscripts for peer review to disseminate these results.

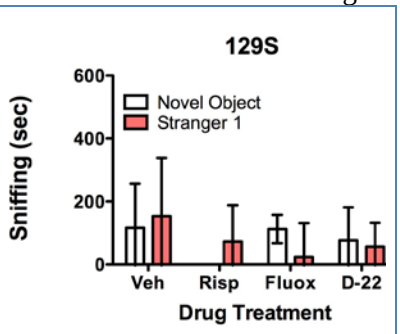

Fig. 5. Partial 129S data showing sniffing in social interaction tests. No preferences for social interaction are evident for any drug treatment due to high variability. N =3-9.

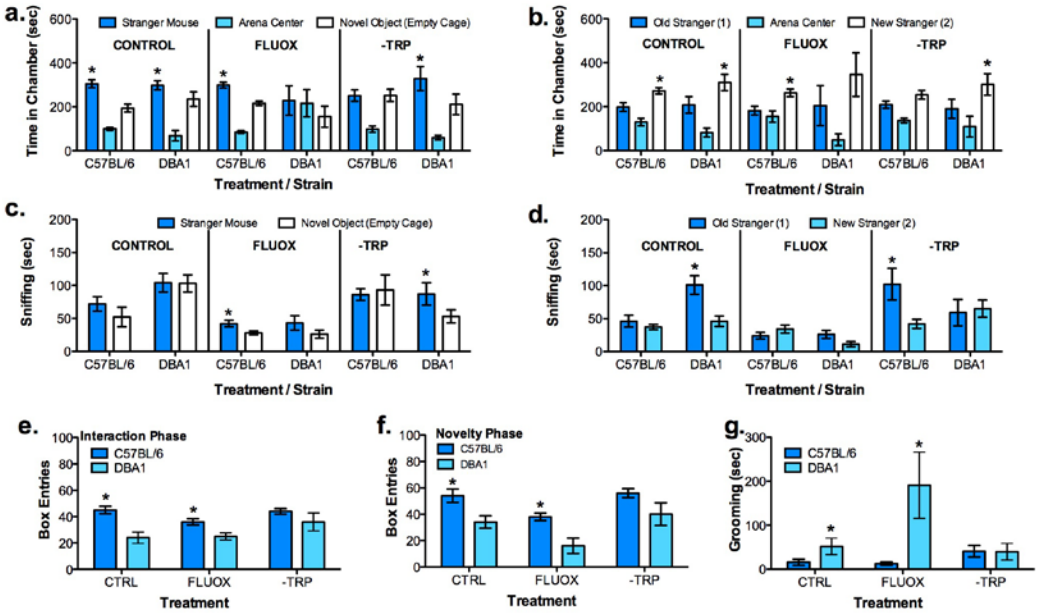

Fig. 6. Social behavior between C57BL/6 and DBA1 mice. (a) Time spent in the chambers of the social interaction test was similar in C57BL/6 and DBA1 mice injected with control vehicle. However fluoxetine impaired sociability in DBA1 mice, while tryptophan depletion (-TRP) impaired it in C57BL6 mice. (b) Both C57BL/6 and DBA1 mice exhibited a preference for social novelty, which was abolished by fluoxetine treatment in DBA1 mice and by –TRP in C57BL/6 mice. Social sniffing during social interaction (c) and social novelty (b) tests were similar among strains, and were reduced by fluoxetine, in this round of experiments neither strain exhibited a preference for social interaction or novelty. C57BL/6 mice made more chamber or box entries in the interaction (e) and novelty (f) phase of the sociability tests. Self grooming (g) was increased by fluoxetine treatment in DBA1 mice, but was otherwise similar among strains. For all graphs *p < 0.05, N = 4-7.

iii. Prepare manuscript and poster for dissemination of results from task 2 (months 25-28)

Poster presentations on Fig. 6 were made at local meetings, and data are complete, further analysis and manuscript preparation by the research group is underway.

Task 3. Serotonin uptake studies in vitro and in vivo in mice:

3a. [^{3}H] serotonin (5-HT) uptake studies in all strains of mice (96 mice)

To measure the ability of D-22 to block [^{3}H] 5-HT uptake *in vitro* [^{3}H] 5-HT saturation uptake experiments were performed *in vitro* in brain synaptosomes harvested from BTBR, C57BL/6 and 129S mice, and maximum velocity of uptake (Vmax) in these strains was 285 ± 16, 297 ± 53, and 303 ± 42 fmol/mg/min and the Km was 31 ± 3, 39 ± 9, 46 ± 13 nM, respectively (**Fig. 7**). In these assays, 20 µM of the SSRI fluoxetine was used to define non-specific binding, which was roughly 30% of total binding. A similar level of non-specific binding was achieved by performing the uptake in the presence of 1 µM of unlabeled serotonin. Sample sizes for these assays range from 4-10 mice/strain. Our overall conclusion is that in vitro 5-HT uptake was not significantly different between the mouse strains we studied, despite previously reported differences in transporter density (Gould et al, 2011) or prior findings of genetic differences in transporter uptake capacity (Carneiro et al., 2009). Indeed the relatively higher expression of serotonin transporter (SERT) in C57BL/6 mice may be a compensatory response to reduced SERT function due to the single nucleotide gene mutation affecting SERT in that strain.

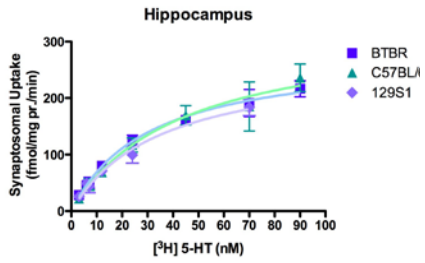

Fig. 7. [^{3}H] 5-HT Uptake saturation in several inbred mouse strains.

b-d. Comparison of D-22 and fluoxetine blockade effects, and their combination and characterize effects of risperidone alone, and with D-22 on 5-HT uptake and test combined effects of D-22, risperidone and fluoxetine on 5-HT uptake (months 5-18)

Competition experiments designed to compare blockade of high-affinity [^{3}H] 5-HT uptake by D-22 to fluoxetine in all four mouse strains were performed over three years in this study. Since co-administration of D-22 with risperidone is of interest as a therapeutic intervention for autism, we also studied that interaction. Representative competition isotherms are shown in **Fig. 8**. Fluoxetine blocks [^{3}H] 5-HT uptake in the nM range, while D-22 blocks it in the low µM range, and also that in general 5-HT uptake is more efficiently blocked by fluoxetine in BTBR and DBA mice than it is in C57BL/6 mice. The reason for relatively inefficient blockade of 5-HT uptake by fluoxetine in 129S mice is not clear. Since risperidone alone has no effect on 5-HT uptake, and because it did not appear to have additive effects with D-22, we did not pursue studies on the interaction between fluoxetine and risperidone on 5-HT uptake. At these concentrations of 5-HT most of the clearance is likely to be due to the presence of SERT, but blockade of uptake 2 transporters by D-22 also appreciably reduced uptake of 5-HT in this context. Since these assays were performed in

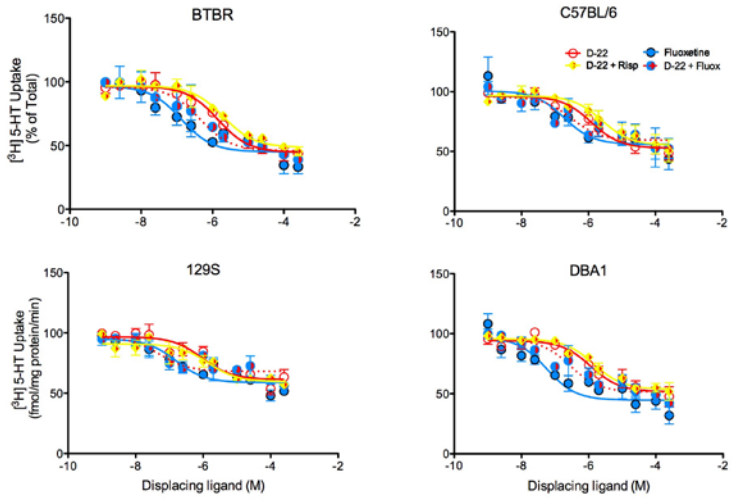

Fig. 8. [^{3}H] 5-HT uptake blockade by risperidone, D-22 and fluoxetine in hippocampal synaptosomes from different strains of inbred mice. Risperidone did not exhibit any appreciable ability to block [^{3}H] 5-HT uptake on its own. Capacity to block 5-HT uptake was at least two orders of magnitude greater for fluoxetine than D-22. Fluoxetine also appeared to work more efficiently in BTBR and DBA mice than it did in C57BL/6 or 129S mice.

mouse tissue, the contributions of norepinephrine or dopamine transporters to the clearance of 5-HT can't be ruled out. In other studies, we observed that D-22 has some capacity to block the norepinephrine transporter in the nanomolar range.

3c. [^{3}H] histamine uptake studies in all strains of mice (96 mice)

In cerebellum from mice, although we did get time-dependent [^{3}H] histamine uptake (Vmax = 17 ± 35 fmol/mg pr./min, Km = 7.327, which is consistent with the only similar prior publication in rat brains (Sakurai et al., 2006). However, we have also observed that ≈ 80% of that is non-specific uptake when defined with 10 uM D-22 (N=3), which may indicate that D-22 has little ability to block the slow rate of histamine uptake in our synaptosomal preparations (**Fig. 9**). In cultured human and rat astrocytes it was reported that histamine uptake rates are an order of magnitude higher than in synaptosomal preparations, and this uptake is blocked to a modest (20-30%) extent by D-22, but not by corticosterone, and that OCT3 plays a relatively minor role to glial histamine uptake in comparison to the plasma membrane monoamine transporter (Yoshikawa et al., 2013; Perdan-Pirkmajer et al., 2012). Hence we tried a Percoll gradient to obtain separate preparations of mouse brain gliosomes and synaptosomes to determine their relative roles in histamine uptake. Through our investigations we found that this approach necessitated a large quantity of mouse brain tissue (from 10 or more) in order to isolate a gliosomal layer. After one such experiment, we did not get appreciably any high affinity saturation binding of [^{3}H] histamine, and non-specific binding was again low. We also utilized SKF 91488 dihydrochloride (Tocris, Bristol, UK) in our assays to reduce enzymatic breakdown of HA. We are now assessing use of metformin, the antidiabetic drug, since it is a high affinity substrate of organic cation transporters (Lee et al., 2014), and have also explored use of [^{3}H] MPP+ to measure uptake 2 clearance capacity. However we have been focused on saturation assays and have not yet measured specific blockade effects of D-22, fluoxetine and risperidone.

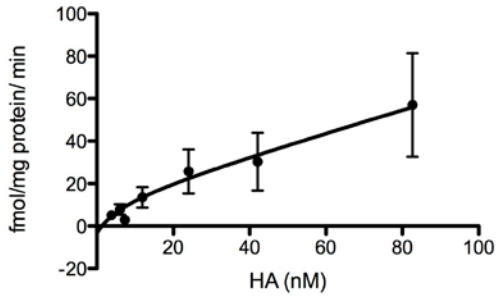

Fig. 9. Histamine uptake in cerebellar synatosomes. Binding was saturable yet the Vmax for uptake is low relative to 5-HT.

3d. In vivo chronoamperometry in BTBR mice to measure 5-HT uptake (96 mice)

a. Pilot studies and histology for electrode placement (months 2-4) This was accomplished in year 1 by thionin staining, electrode placement in hippocampus and frontal cortex was accurate, even in BTBR mice with impaired hippocampal structure.

b. Serotonin clearance with selective pharmacological blockade (months 5-18)

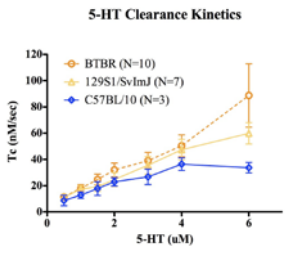

Fig. 10. 5-HT clearance in hippocampal CA3.

In vivo, we examined clearance of exogenously applied 5-HT locally into the CA3 region of the hippocampus of anesthetized mice by pressure-ejection using high-speed chronoamperometry. Decay of the resultant oxidation current was detected by a Nafion-coated carbon fiber electrode in close proximity to the pipette. The linear slope of the oxidation decay curve from 20% to 60% of maximal signal amplitude (Tc) was the parameter compared among strains as an index of 5-HT clearance rate (**Fig. 10**). Tc values were comparable among strains at low 5-HT concentrations, but it appears that 5-HT clearance may be more rapid in BTBR and 129S than in C57BL mice at high, but physiologically relevant 5-HT concentrations. So when lesser amounts of 5-HT were applied, in the range of 100 nM -4 µM, clearance rates were similar. However, when 6 µM was applied, 5-HT clearance in C57 was slower than in BTBR or 129S mice.

c. Serotonin clearance following acute systemic drug administration (months 21-32)

In BTBR mice, the effects of systemic (i.p.) injection of D-22 on in vivo serotonin clearance were examined in the anterior cingulate cortex. The stereotaxic coordinates for recording were [from Bregma]: AP (+0.7mm); ML (+0.15mm); DV (from dura: (-0.75mm)). D-22 was injected after recording had begun, and serotonin was exogenously applied and recorded over four time intervals as shown in **Fig. 11.** The barrel concentration of serotonin was 200uM and the volume delivered ranged from 25-250nL. The amount ejected was 5-50 pmols, and mice were recorded from for roughly 2 hours. We observed that systematically injected D-22 (0.01 mg.kg) slowed the clearance of 5-HT in this brain region to a significant extent. This suggests that D-22 administration as we have used in our behavior studies does significantly prolong the amount of time that 5-HT is present in extracellular fluid, and also perhaps in the synapses.

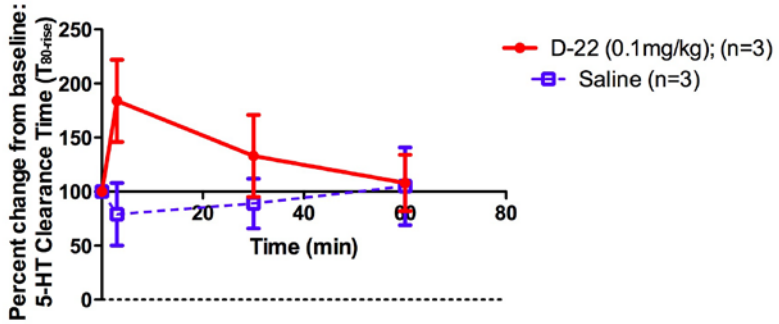

Fig. 11. Effects of systemic D-22 administration on serotonin clearance capacity in the anterior cingulate cortex. Systemically injected D-22 slows the clearance of serotonin (5-HT) from brain extracellular fluid as compared to systemic saline injections.

3e. Western blot and autoradiography for OCT3 and SERT expression in mouse brain (months 20-34)

Coronal sections (20 um) were taken in frontal cortex, hippocampus and amygdala regions of fresh frozen brains from 5 and 16 week old C57, BTBR and 129S mice. The sections mounted onto gelatin-coated slides, desiccated for 18-24 h at 4°C stored at -80°C. Prior to experiments sections were thawed for 1h at 4°C. They were then pre-incubated for 20 min in 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl pH 7.4 at ~26°C. Incubation in a slide mailer (10 ml) for 1 hour at room temperature in buffer + 40 nM [^{3}H] D-22 (ARC, St. Louis, MO), plus 25 nM mazindol and 25 nM sertraline to block binding to uptake 1 monoamine transporters. Based on saturation binding in membrane homogenates, occupancy of binding sites at this concentration of [^{3}H] D-22 = 86%. Non-specific binding was defined by 100 uM D-22, and was 50% of total binding. Incubation was terminated by two 10 min washes in 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl pH 7.4 at 4°C, followed by a 5 sec dip in de-ionized water at 4°C. Slides were dried on a slide warmer for 1 hour. [^{3}H]D-22 labeled sections on slides exposed to Kodak MR film for 66 h, with ^{3}H standards previously calibrated to brain mash containing known quantities of ^{3}H ligand. Autoradiogram images on film were captured on a digital imaging system. In sum, as in **Fig. 12** we observed no significant differences among strains or ages in D-22 binding sites in the cortex. Since non-specific binding was so high, we may further refine this assay to reduce this potentially confounding factor in future assays.

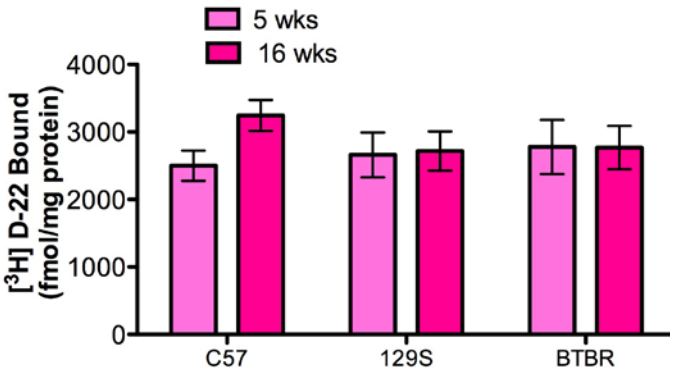

Fig. 12. [^{3}H] D-22 specific binding in 5 or 16 week old mouse cortex. We did not find significant differences between strains or ages, although values in 16 week old C57 mice tended to be elevated, and non-specific binding was 50% of total, N= 8 mice per treatment group.

Western blotting for OCTs and serotonin transporters in the brain was not possible despite our efforts to measure it in the mouse brain or in heterologous cells. An experienced post-doctoral fellow tried several commercially available and custom antibodies, and unfortunately they provided false indication that OCT3 was present in OCT3 knock out mice at levels equal to wild-types. Four different commercially available antibodies for OCT3 were utilized. Proper experimental techniques were observed for gel loading and all data were normalized to β-actin protein expression. A 1.5 year effort was dedicated towards experiments to detect transporter expression via western blotting. The experiments were terminated due to non-specificity of OCT3 protein detection despite use of multiple t sources of primary antibodies.

3f. Prepare manuscripts and poster presentations to disseminate results (months 35-36) We have sufficient data available to prepare manuscripts to disseminate these results of task 3. Manuscript preparation is underway and once complete will be submitted to peer-reviewed journals for publication.

Task 4. Measuring the effects of treatment with D-22 and/or other common drug treatments for autism, on mouse social interaction and social dominance, grooming and marble burying behaviors: 4a. Strain characterization of social interaction, grooming behavior and marble burying (360 mice): Baseline strain comparisons of social interaction, grooming behavior during sociability tests and marble burying behavior were made at 5 and 16 weeks of age in male mice. We found that BTBR and 129S1 mice exhibit impaired social behavior relative to C57BL/6 or DBA1 mice, but only BTBR mice bury more marbles than other strains. These data were published in Gould et al., (2014).

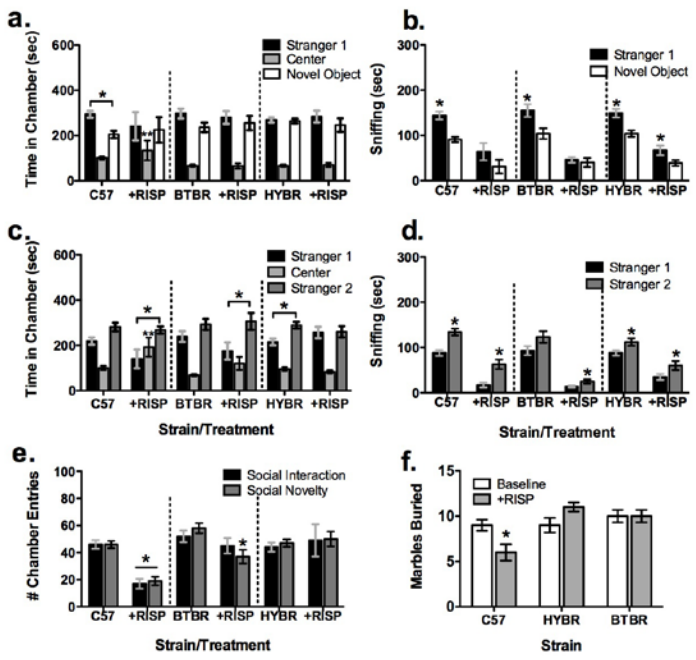

Fig. 13. Behavior of mice in autism-relevant tests. Control and risperidone (+ RISP, 0.01 mg/kg) treated male BTBR, C57BL/6 mice and their F1 hybrids were examined. (a) Only C57BL/6 mice had a preference for interaction as indicated by significantly more time spent in the chamber with the stranger mouse than novel object (*p <0.05), but this preference was not displayed by C57BL/6 mice given an i.p. injection of risperidone; they instead spent more time in the center (**p < 0.05). (b) All strains exhibited a baseline preference for social interaction (*p <0.05), which was abolished in all but hybrids by risperidone. (c) Naïve hybrids and risperidone-treated BTBR and C57BL/6 had chamber times indicative of preference for social novelty (*p <0.05). (d) C57BL/6 and hybrid mice, and all mice treated with risperidone had significant social novelty preference (*p <0.05) when sniffing time was monitored, although sniffing time was diminished in risperidone-treated mice (b and d). (e) Total chamber entries and (f) the marbles buried after sociability tests was significantly reduced by risperidone treatment in C57BL/6 mice (*p <0.05, N = 7-19).

i. Single drug treatments (months 2 – 12)

The atypical antipsychotic risperidone is often used in the treatment of autism to control aggression, yet typically it does not improve social interaction behavior. Corresponding with this, previously we found that risperidone (0.1 mg/kg) failed to improve social behavior, but it reduced mobility and marble burying in BTBR mice (Gould et al., 2011). Hence we examined a lower dose in these three mouse lines, and found that risperidone (0.01 mg/kg) worsened social behavior in C57BL/6 mice, but had no effect in BTBRxBL6 F1 hybrid or BTBR mice when chamber entries were considered (**Fig. 13**). In general it appeared that the dose of 0.01 mg/kg of risperidone inhibited locomotor activity in C57BL/6 mice generally, as evidenced by their reduced chamber entries during the test and marble burying behavior after the test was completed. Hence C57BL/6 may be more sensitive to the effects of risperidone than either BTBR or hybrid mice. Based on this and the monoamine uptake data that we have generated, we are in the process of preparing a manuscript to submit for peer-review featuring this social behavior data of the hybrid as a key component. More recently we treated BTBRxBL6 F1 with D-22 and observed that it enhanced sociability in these mice in a similar manner to results observed with BTBR, and marble burying was reduced.

We also examined the ability of selective serotonin reuptake inhibitors to enhance sociability in BTBR mice. Since fluoxetine had already been tested in BTBR mice, we decided to test the effects of citalopram on social behavior. We found that citalopram administration at a range of doses failed to enhance sociability (Fig. 14.).

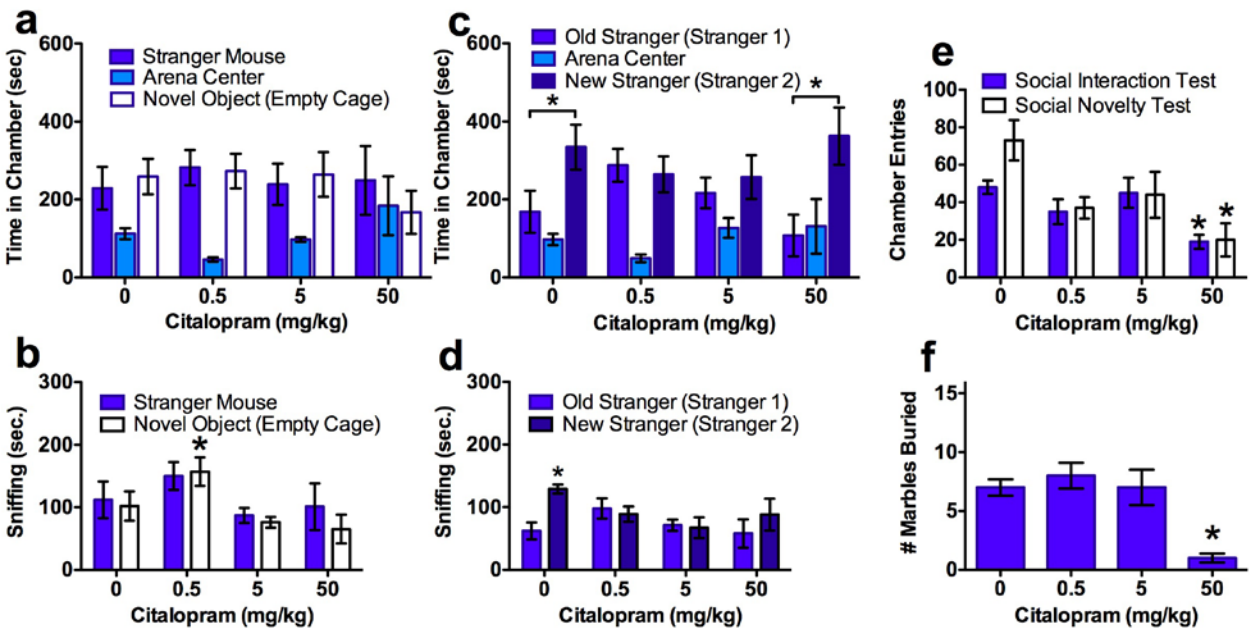

Fig. 14. Dose-dependent effects of citalopram on BTBR sociability preferences, locomotor activity and repetitive behavior. Citalopram i.p. injection did not promote (a) dwelling by or (b) sniffing of stranger mice. Also citalopram treatment resulted in a loss of social novelty preference in chamber dwelling (c) with the exception of the highest 50 mg/kg dose, and also reduced (d) social sniffing of novel mice. The dose of 50 mg/kg impaired locomotor activity (e) as evidenced by fewer box entries and also (f) reduced repetitive marble burying behavior (N=6-8). These findings are in stark contrast to what we have observed previously with fluoxetine or vortioxetine administration in BTBR mice. This finding demonstrates some selective serotonin reuptake inhibitors fail to enhance sociability, which supports the idea that SSRIs vary in their effects, possibly due to differences in affinity for SERT or in where on the SERT their binding sites are.

Additionally, in researching other treatments in common medical use that are blockers of OCTs, we came across berberine as an alternative for D-22. We have already established that in three-chamber sociability tests the uptake 2 blocker decynium-22 (D-22) improves social behavior in otherwise socially-impaired BTBR T+/tf mice. The alkaloid antibiotic berberine also has antidepressant-like properties in mice, and is both a blocker and substrate of OCTs. Given this, we hypothesized berberine might improve the sociability of BTBR mice as D-22 does. Indeed, berberine significantly increased BTBR preference for social interactions and social sniffing ($p < 0.05$, N=8-9). Furthermore it enhanced the already sociable behavior of C57BL/6 mice, but it had no impact on repetitive marble burying in either strain. Our findings confirm that systemic OCT3 and uptake 2 blockade is a promising strategy for improving social behavior warranting further investigation as a treatment for sociability impairments. T

ii. Combined drug and behavior comparative studies (months 13-24)

We have been examining the interaction between D-22 and risperidone, with the idea that the effects of D-22 co-administration might augment the ability of risperidone to combat both of the core symptoms of autism. While risperidone is effective at reducing marble burying, it is generally not able to enhance social behavior (Gould et al., 2011). D-22 complements risperidone because it is effective at promoting social behavior, yet it is generally ineffective at reducing marble burying behavior in mice. So far we have examined in BTBR mice if combined D-22 (0.01 mg/kg) and risperidone (0.01 mg/kg will effectively reduce sociability impairments and improve social interaction. The combined therapy appears to be effective at these doses.

4d. Strain characterization of social dominance and drug effects (192 mice):

We used the tube test for social dominance to examine the effects of D-22 on impulsive social behavior. These tests are performed in a tube that is sufficiently narrow that mice can't turn around in them, nor crawl over or under one another. No pre-conditioning of subjects takes place prior to testing, and 129S1/SvImJ mice are used as stimulus 'competitors'. The test starts when mice meet in middle of the tube and the barrier is removed. The test ends when one mouse's back feet touch ground or after 3 minutes. Mice are scored as follows: Win = 1; Loss = 0; Draw = 0.5. For each mouse a minimum of 6 rounds of testing is performed, with a 10 min rest between each match. We were able to examine the behavior of C57BL/6 mice on D-22 or fluoxetine as compared to vehicle controls using the tube test, and as shown in **Fig. 15** we observed a dose-specific increase in impulsive behavior, as evidenced by an increased number of advances and wins in those treatment groups. In BTBR and 129S mice we did not observe a parallel increase in social dominance with D-22 at 0.1 mg/kg and we have yet to examine the effects of SSRIs. In DBA1 mice there was no effect of fluoxetine on social dominance. Thus it appears that social dominance is more responsive to drug treatment in C57BL/6 mice than in these other strains. Further tube tests are underway to confirm these findings. However it seems that strains with more overtly dominant behavior such as 129S have less sensitivity to drug treatments in these tests.

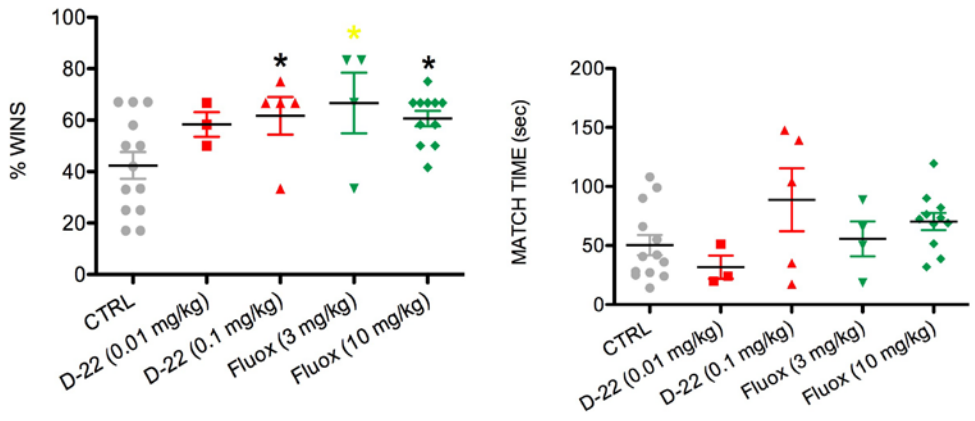

Fig. 15. Effects of systemic D-22 administration on social dominance in C57BL/6 mice. Systemically injected D-22 (0.1 mg/kg) increased wins in the social dominance tests in a similar manner to fluoxetine, without increasing the duration of the tube test matches.

4e. Measurement of serum levels of drugs from comparative studies (months 9 – 36)

Concentrations of D-22 have been measured in serum, but concentrations of risperidone and fluoxetine have not yet been measured, however plans are in place to measure drug concentration in these samples.

4f. Collect brains from vehicle treated mice for westerns and autoradiography (months 9-36) Tissue was collected and some of the findings are reported in Fig. 12, other samples remain frozen at -80ºC ready for analysis once better antibodies become available for OCT3 and SERT in mouse tissues.

Task 5. Meet reporting requirements, disseminate results and seek continued support for research: Quarterly research meetings were held in year one and two, and biannual meetings were held in year 3. Meetings will continue for the purpose of manuscript preparation. Dr. Gould and Dr. Daws have attended at least one annual meeting per year to disseminate results, and their students and research assistants have also presented data at meetings. Dr. Gould and her colleagues are continuing to work on preparing 2 to 3 manuscripts to submit to peer-reviewed journals for review and publications. No human data was collected so we do not have any relevant data pertinent to the mission of NDAR to submit.

5e. **Future studies**: We have conducted chronic administration of D-22 in BTBR and C57BL/6 and have found their sociability promoting properties are similar to acute effects. We are preparing proposals to seek funding from NIH, private organizations and other DOD mechanisms for autism research to continue this line of research. Of immediate interest are the effects of metformin at OCT3 and its ability to counteract adverse metabolic effects of chronic risperidone treatment.

KEY RESEARCH ACCOMPLISHMENTS:

- Task 1: Institutional regulatory approvals granted for proposed research, mouse colonies established and producing sufficient numbers for experiments in each year of the study

- Task 2,Pharmacokinetic studies of D-22 in serum and brain were completed for 10, 1, and 0.5 mg/kg, by HPLC and GC/MS are complete or nearly complete

- Task 2, aim 3 social behavior studies examining the effects of D-22 alone, in comparison to fluoxetine and risperidone, or combined with fluoxetine and risperidone are complete 4 strains of mice. The 3-chamber sociability tests and tube tests for social dominance seem to be providing much needed insight for translational studies.

- Task 2, Serum corticosterone measures as they relate to behavior testing, mouse strain and drug treatment are complete

- Task 3 [^{3}H] serotonin uptake protocol is finalized, and competition assays performed in all mouse strains.

- Task 3In vivo chronoamperometric comparisons of 5-HT clearance in the CA3 of hippocampus have been made among inbred strains of mice, and acute effects of D-22 on clearance of 5-HT in the brain were observed.

- Task 4. Combined D-22 and risperidone and D-22 and fluoxetine behavior and in vitro uptake experiments were performed. The acute effects of D-22 have been measured in dominance tube tests, marble burying tests and grooming behavior tests.

- Task 5. Progress report meetings have been held every 3 months. The PI , Co-I and research staff have been presenting poster on some of these findings at different annual meetings. Some manuscripts have been published and others are under preparation.

REPORTABLE OUTCOMES:

Employment and Career Promotion Based on the Award

Georgianna Gould, PI, was promoted to an associate professor, research track. Dr. Gould was interviewed by Baylor University for a tenure-track assistant professor position in the psychology department, and was the second choice candidate. She was also considered for a tenure track position at the University of Texas at San Antonio. Finally Dr. Gould was invited to review abstracts for the INFAR autism meeting and to serve as an ad-hoc member of a committee reviewing grants for the National Science Foundation.

Lyn Daws, Co-I was promoted to full Professor and is endowed by Frost Bank.

Corey M. Smolik, a former University of Texas at San Antonio Biology undergraduate student, who was interning in the lab under the University of Texas Health Science Center START UPs program (R25 GM097632) was hired as a full-time research assistant by Dr. Gould to analyze videos of social behavior and to assist with performance of uptake assays. He was taught to perform the uptake assays, and radioligand binding this year and performed these experiments proficiently. He subsequently took Emergency Medical Technician training, and in 7/2014 he got a job at the University Hospital as an emergency room technician. He also attended the Experimental Biology Meeting in 2014 to present this work. He was admitted to the Texas Tech University Health Science Center as a student Physician Assistant Program.

Wynne Q. Zhang, a freshman at Rice U. served for years as a high school and college level undergraduate student researcher in the lab for the past 3 years. This should provide helpful with her medical school or graduate school applications and provide her with the confidence and experience she needs to successfully apply for graduate school.

Sergey Poplyaev was hired as a Research Laboratory Technician to replace Corey Smolik in March 2015. He managed mouse colonies and learned to perform three chamber tests, but resigned from this position on June 23, 2015 due to the demands of the job being too great.

Marshall Edwards was hired on May 4, 2015 as a research assistant and quickly took over mouse colony management, behavior tests, video analysis and is now learning to perform uptake and binding assays in vitro. He was accepted into a master's program in immunology and infection at UTHSCSA and now has dual appointment as a student and research assistant. He has won several awards for his poster presentations.

Clover Moten a high school student from San Antonio who worked on this project as part of her Voelcker Foundation Scholarship, she graduated this year and will attend college in the fall.

Alicia Sanchez graduated from St. Mary's University and is taking a year off to perform missionary service for her church. As a student researcher in the lab, she performed work on this project and made poster presentations to disseminate results at local meetings. Next year she will apply for graduate school, and her experience through this program and authorship on manuscripts should strengthen her candidacy.

Benita Lee is a high school student from San Antonio who worked on this project as part of her Voelcker Foundation Scholarship, she has returned this summer to continue working on manuscripts from this project.

Research Oral Presentations:

7/2014 Voelcker Scholars Research Presentation, UT Health Science Center, San Antonio, TX "Mouse Behavior Models in Autism Research"

12/2013 Dept. of Neurobiology and Psychology, Baylor University, Waco TX "A second look at serotonin uptake blockade to improve social behavior"

12/2015 Dept. Biology, William Paterson University, Wayne NJ, "Next Generation Uptake Inhibitors: Targeting Ancillary Monoamine Transporters"

10/2015 Chicago IL, Society for Neuroscience, #467, Hormones, Neurotransmitters & Social Behavior, "Uptake 2 Transporter Blockade Ameliorates Deficits in Sociability in Two Mouse Models"

10/2015 Dept. of Physiology, UT Health Science Center, San Antonio, TX, "Sustained Uptake 2 Blockade and Sociability in an Insulin-Resistant Mouse Model of Autism"

7/2015 Voelcker Scholars Research Presentation, UT Health Science Center, San Antonio, TX "Serotonin's Roles in Shaping Social Behaviors"

3/2015 UTSA Biology, San Antonio, TX "Serotonin as a key shaper of social behavior"

10/2015 UTHSCSA "Sustained Uptake 2 Blockade and Sociability in an Insulin-Resistant Mouse Model of Autism"

Abstracts and Poster Presentations:

Program#: 547.01 Poster#: OO16 Author: Georgianna Gould Title: Targeting serotonin uptake to ameliorate social behavioral deficiencies in pre-clinical models. Presentation Time: Tuesday, Nov 12, 8:00 AM – 9:00 AM, Society for Neuroscience annual meeting, San Diego, CA, Nov 9-13, 2013.

Abstract #920. Smolik C., Javors M., Hensler, J.G., Koek, W. Daws, L.C., Gould G.G. (2013). Effects of "Uptake 2" Blockade and/or Risperidone on Murine 5-HT Uptake, Social and Repetitive Behavior. Poster presentation, 52nd Annual Society of Toxicology Meeting, San Antonio, TX March 10-14. In the Toxicologist (Supplement to Toxicological Sciences) Vol 132, issue 1.

Mitchell, N, Owens W, Horton R, Vitella M, Gould G, Koek W, Daws L. 2013. Mechanisms contributing to lack of antidepressant efficacy in juveniles and adolescents. Society for Neuroscience Meeting, San Diego, Poster # 227.12/E21.

Gould, GG 2013. Targeting serotonin uptake to ameliorate social behavior deficiencies in pre-clinical models. Society for Neuroscience Meeting, San Diego, Poster # 547.01/OO16.

Smolik, CM, Zhang WQ, Vitela M, Sanchez JJ, Javors MA, Koek W, Daws, LC, Gould, GG. 2014. Blockade of serotonin uptake by decynium-22 enhances social behavior. Experimental Biology 2014, San Diego, Poster #14-3827-EB.

Gould, GG 2014. "Decynium-22 Enhances Social Behavior in Serotonin Transporter Knock-Out Mice. American College of Neuropsychopharmacology, 53rd Annual Meeting, Phoenix, AZ, December 10, 2014. Poster # W188.

Sanchez A, Smolik C, Pham T, Lalani K, G. Gould. Berberine blocks 'uptake 2' and enhances mouse sociability. UTHSCSA Mikitin Research Forum, San Antonio, TX, May 11, 2014.

Gould, GG 2015. "Uptake 2 Transporter Blockade Can Ameliorate Sociability Deficits", International Meeting for Autism Research, Salt Lake City, UT, May 15,2015 Poster # 139.149, Session: Interventions Pharmacologic.

Gould, GG 2016 "Effects of Pargyline and Para-Chlorophenylalanine on Mouse Social Behavior" International Meeting for Autism Research. Baltimore, Abstract ID#23062.

Gould G "Influence of Central Serotonin Availability on Social and Repetitive Behavior in Mice" Abstract #1319 Experimental Biology 2016 Meeting San Diego.

Manuscripts:

Gould GG, Burke TF, Osorio MD, Smolik CM, Zhang WQ, Onaivi ES, Gu TT, DeSilva MN, Hensler JG. 2014. Enhanced novelty-induced corticosterone spike and upregulated serotonin 5-HT1A and cannabinoid CB1 receptors in adolescent BTBR mice. Psychoneuroendocrinology. 39:158-69. doi:10.1016/j.psyneuen.2013.09.003.

Zhang WQ, Smolik CM, Barba-Escobedo PA, Gamez M, Sanchez JJ, Javors MA, Daws LC, Gould GG. 2015. Acute dietary tryptophan manipulation differentially alters social behavior, brain serotonin and plasma corticosterone in three inbred mouse strains. Neuropharmacology. 2015 Mar;90:1-8. doi:10.1016/j.neuropharm.2014.10.024.

Horton RE, Apple DM, Owens WA, Baganz NL, Cano S, Mitchell NC, Vitela M, Gould GG, Koek W, Daws LC. 2013. Decynium-22 enhances SSRI-induced antidepressant-like effects in mice: uncovering novel targets to treat depression. J Neurosci. 33(25):10534-43. doi: 10.1523/JNEUROSCI.5687-11.2013.

Mitchell NC, Gould GG, Smolik CM, Koek W, Daws LC. 2013. Antidepressant-like drug effects in juvenile and adolescent mice in the tail suspension test: Relationship with hippocampal serotonin and norepinephrine transporter expression and function. Front Pharmacol. 4:131. doi: 10.3389/fphar.2013.00131.

Mitchell NC, Gould GG, Koek W, Daws LC. 2016. Ontogeny of SERT expression and antidepressant-like response to escitalopram in wild-type and SERT mutant mice. J Pharmacol Exp Ther.. pii: jpet.116.233338.

Manuscripts under review or requiring revision and resubmission:

Sanchez A, Smolik CM, Pham TV, Lalani K, Gould GG. Sociability of Two C57BL/6 Mouse Substrains from Different US Suppliers. J Vet Behavior

Lee B, Pehrson A, Witt N, Allen J, Sanchez C, Gould GG. Vortioxetine inhibits marble burying and transiently enhances social sniffing in adult male BTBR mice. Autism Research, 15-Dec-2015.

Other Products:

In addition to other oral presentations and posters reported in the "Dissemination of Results" the PI will chair a symposium at the "Serotonin in Seattle" meeting in July 2016. The title of the symposium will be "The highs and lows of serotonin in autism spectrum disorders". The meeting is hosted by the International Society of Serotonin Research.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

1. Project Director/Principal Investigator

Name: Georgianna Gould
Project Role: Principal Investigator
Researcher Identifier: gouldg (ERA Commons)
Nearest person month worked: 4.8 mos/yr, will change to 1.2 mos/yr for extension
Contribution to Project: Dr. Gould is the PI on the project and is responsible for performing or overseeing the performance of all aspects of the project.

W81XWH-12-1-0506 (Gould, PI) 09/30/12 – 03/31/2016
Autism Idea Award AR11019 CDMRP/DOD Annual Direct
Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders
The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Gould, Other Support:
Role: PI, 0.12 mos/yr
Lindow, Stevens & Treat Research Award, 2/1/2012-7/30/2016,
Title: Neuroprotection from pesticide-induced sensitization via transporter blockade
Goals of project: To examine in zebrafish if up-regulation of uptake 2 transporters contributes to pesticide-induced sensitization to neurotoxins, and if their blockade is neuro-protective.

Role: PI, 2.4 mos/yr
NIH R21HD081261-01 (Gould), 4/1/2015-3/31/2017
Title: Impact of Gestational Serotonin Availability on Brain Function & Social Behavior
Goals of project: To study the impact of limiting gestational serotonin availability on brain development and social behavior.

Role: Collaborator, 1.9 mos/yr
5R01MH093320-02 (Co-PIs Daws/Koek), 3/1/2012 – 11/30/2016
Title: Organic cation transporters as targets for novel antidepressant drugs
Goals of project: To examine the efficacy of OCT3 blockade as an antidepressant in vitro and in vivo.

Role: Collaborator, 3.24 mos/yr
1R01MH106978-01 (PI Daws), 5/1/2015 – 11/30/2016
Title: Age-related differences in serotonin clearance: Novel targets for antidepressants
Goals of project: To investigate expression and activity of transporters for serotonin, including the high-affinity serotonin transporter (SERT), as well as the low-affinity, high-capacity organic cation transporters (OCTs) and plasma membrane monoamine transporter (PMAT), in juvenile and adolescent mice.

2. Other Effort-Contributing Researchers

Name: Marshall Edwards
Project Role: Research Assistant
Nearest person month worked: 6 mos/yr

Contribution to Project: Assisted Dr. Gould by maintaining mouse colonies, performing radioligand uptake and binding assays, performing ELISA assays for serum corticosterone, and collecting data from behavior videos.

Other Support: Dr. Gould's LST Research Award
Nearest person month worked: 6 mos/yr
Contribution to Project: Perfomed radioligand binding assays, repaired aquatic habitat

Name: Alicia Sanchez
Project Role: Undergraduate Research Assistant
Nearest person month worked: 1 mos/yr
Contribution to Project: Assisted Dr. Gould by measuring autoradiograms, performing behavior tests and collecting data from behavior videos.

Other Support: None

Name: William Anthony Owens
Project Role: Senior Research Associate
Nearest person month worked: 0.12 mos/yr
Contributions to Project: Performed in vivo chronoamperometry in BTBR mice.

Other Support: 5R01MH093320-02 (Co-PIs Daws/Koek)
Contribution to Project: In vivo chronoamperometric recordings.
Name: Lynette C. Daws
Project Role: Co-Investigator
Researcher Identifier: daws (ERA Commons)
Nearest person month worked: 1.2 mos/yr
Contribution to Project: Dr. Daws oversees performance of chronoamperometry and makes intellectual contributions to uptake assays she also makes her laboratory space and personnel available for these studies.

W81XWH-12-1-0506 (Gould, PD/PI) 09/30/12 – 09/29/2015
Autism Idea Award AR11019 CDMRP/DOD Annual Direct
Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders
The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Daws Other Support:
1R01 MH106978-01 (Daws) 05/2015 – 03/2019 3.6 calendar months
NIH/NIMH
Age-related differences in serotonin clearance: Novel targets for antidepressants.
These studies investigate the expression and activity of transporters for serotonin, including the high-affinity serotonin transporter (SERT), as well as the low-affinity, high-capacity organic cation transporters (OCTs) and plasma membrane monoamine transporter (PMAT), in juvenile and adolescent mice. These studies take advantage of mice with constitutive reductions or knockout of SERT, OCT3 or PMAT to understand mechanisms contributing to serotonin clearance in brain during these young ages, with the goal to understand the mechanistic basis for lack of therapeutic efficacy of SSRIs in juvenile and adolescents who suffer depression, and to identify new targets for the development of antidepressants with improved therapeutic efficacy for this young population.
Role: Principal Investigator

1R01 MH093320-01 (Daws and Koek) 03/2012 – 11/2016 3.6 calendar months
NIH/NIMH
Organic cation transporters as targets for novel antidepressant drugs
Experiments in this study are designed to validate organic cation transporter-3 (OCT3) as a target for the discovery of drugs with improved therapeutic potential for the treatment of depression in adults. In these studies we test analogs of

decynium-22, the prototypical blocker of OCTs. These analogs vary in their affinity for OCT3 and will be used, together with OCT3 KO mice, to investigate the contribution of OCT3 to serotonin, norepinephrine and dopamine uptake in adult brain, and the consequence of their blockade for antidepressant-like behavior.
Role: Co-Principal Investigator

1R21 DA038504 (Daws) 06/2014 – 05/2016 2.4 calendar months
NIH/NIDA

The dopamine transporter in eating disorders: Uncovering novel therapeutic targets.
Eating disorders, including anorexia, bulimia nervosa and binge eating, are major public health concerns, particularly prevalent in adolescent girls, and are compounded by a lack of effective treatments. Dysfunction of dopaminergic neurotransmission is implicated in these illnesses, but studies investigating the relationship between the dopamine transporter, a primary regulator of dopamine neurotransmission, and eating disorders during adolescence and adulthood are lacking. These studies will begin to fill critical knowledge gaps, with the long-term goal to elucidate novel targets for the treatment of these debilitating and often fatal disorders.
Role: Principal Investigator

R13 DA033783 (Daws) 04/2012 – 03/2017 0.12 calendar months
NIH/NIDA
Serotonin Club Meetings 2012-2016
This award provides funds to offset travel expenses for young investigators (students, post-docs and junior faculty not more than three years past their post-doc) to attend the 2012, 2014 and 2016 meetings of the International Society for Serotonin Research (ISSR), formerly, the Serotonin Club. There is no salary commitment.

R01 DA026947 (Khoshbouei) 07/2015 – 04/2017 0.36 calendar months
NIH/NIDA (subcontract component)
Methamphetamine regulates the dopamine transporter via an intracellular mechanism
These studies investigate sigma receptor regulation of the dopamine transporter using in vivo electrochemical approaches.
Role: Principal investigator of subcontract

Center for Biomedical Neuroscience Pilot Grant (Paukert)
01/2015 – 12/2015 0.12 calendar months
University of Texas Health Science Center
Electrochemical tracking of behavioral state-dependent neuromodulation.
This pilot study aims to develop fast-scan cyclic voltammetry to measure norepinephrine release in primary visual cortex in head-fixed, awake mice following ambulatory and visual stimulation.
Role: Co-Investigator

Name: Martin A. Javors
Project Role: Collaborator
Researcher Identifier: javors (ERA Commons)
Nearest person month worked: 1.2 mos/yr
Contribution to Project: Dr. Javors measures tissue levels of decynium-22 and other drugs for pharmacokinetic studies, and also measures brain levels of monoamines by HPLC.

W81XWH-12-1-0506 (Gould, PD/PI) 09/30/12 – 09/29/2015
Autism Idea Award AR11019 CDMRP/DOD
Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders
The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Javors Other Active Support:

R01-AA022361(co-PIs: Javors/ Dougherty) 9/2013 - 6/2017
NIAAA Role: Co-Principal Investigator (20%) – 2.4 months
Title: Phosphatidylethanol and Other Ethanol Consumption Markers
This study is designed to characterize and validate phosphatidylethanol as a biomarker for alcohol consumption and then determine how it alone (and in combination with 3 other alcohol biomarkers) can be used to identify an individual's level and pattern of drinking.

P30AG013319-20 (PI: Javors) 6/1/2014 – 6/30/2015
NIA Role: Principal Investigator (3%)- 0.36 months
Title: Bioanalytical Pharmacology Core, Nathan Shock Center, Barshop Institute
The purpose of the center is to provide analytical support to research groups for aging studies. Measurement of drug levels in dosage forms (usually food) and animal blood and tissues is provided in addition to assistance with study design is offered.

5UO1-AG022307-09 Strong (PI) 09/01/2014 - 08/31/19
NIA Role: Co-Investigator (30%)- 3.6 months
Center for Testing Potential Anti-aging Interventions
The purpose of the center is to participate in a cooperative study to test interventions for which therapeutic targets have been identified that have been shown to control the aging process.

3R01-AA014988-11S2/NIH Dougherty (PI) 05/10/14 - 03/31/15
NIMH Role: Co-Investigator (8%)-0.96 months
Impulsivity and Biological Markers for Suicidality and Drug Use in Adolescents
This 5-year longitudinal study is designed to examine the interrelationships among impulsivity, 5-HT, stressful life events and the outcomes of drug use and suicidality in high-risk adolescents.

2R01-MH076929-06A1 Xin-Yun Lu (PI) 04/01/06 - 07/31/17
NIMH Role: Co-Investigator (5%)-0.6 months
Characterization of leptin's antidepressant activity
The goals of this renewal project are to determine the key components of glutamate neurotransmission that are responsive to leptin signaling and are responsible for the antidepressive effects of leptin.

1R21-MH097092-01 Bowden (PI) 04/25/12 - 03/31/16 (no cost extension)
NIMH Role: Co-Investigator (2%)-0.24 months
Calcium Study of Lymphoblasts in Bipolar Patients to Aid Diagnosis and Treatment
The overall goals of this project are to study the regulation of calcium activity in immortalized lymphocytes (LCLs) to develop a biological component as part of the diagnoses of bipolar disorder, resulting in more personalized, effective treatments and outcomes of bipolar disorder.

W81XWH-08-2-0117 PTSD MRC Roache (PI) 09/01/08 - 08/31/15
DOD-CDMRP- STAR PTSD Res Cnsrtium Role: Co-Investigator (8%)-0.96 mos
SSRI Treatment of Alcohol PTSD Dual Diagnosis – A Test of the Serotonin Hypothesis
This is an outpatient treatment trial evaluating the clinical utility of alcoholism subtyping to predict response to the use of sertraline to treat OEF/OIF veterans with dual diagnosis.

Name: Wouter Koek
Project Role: Collaborator
Researcher Identifier: koek (ERA Commons)
Nearest person month worked: 0.6 mos/yr
Contribution to Project: Dr. Koek provides space for animal behavior studies and statistical consultation and other intellectual contributions as needed.

W81XWH-12-1-0506 (Gould, PI) 09/30/12 – 09/29/2015
Autism Idea Award AR11019 CDMRP/DOD

Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders
The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Koek Other Active Support:
1R01 MH093320 (Daws, Koek) 03/01/12 – 11/30/16
NIH/NIMH 3.6 cal mo
Organic Cation Transporters as Targets for Novel Antidepressant Drugs
The results of the studies under this grant will help to establish OCT3 (and/or PMAT) as a novel target for the discovery of drugs with improved therapeutic potential, as well as provide a mechanism that can, at least in part, account for poor therapeutic response to current antidepressant drugs.

R01 DA05018 (France) 03/15 – 03/20
NIH/NIDA
Discriminative Stimulus Effects of Opioid Withdrawal
This grant examines interactions between morphine and serotonergic (e.g., fluoxetine) or cannabinoid (e.g., THC) drugs to determine whether the combination enhances their ability to alleviate pain without increasing, and possibly decreasing, their abuse and dependence.

R01 MH106978-01 (Daws) 05/15 – 05/20
NIH/NIMH
Age-related differences in serotonin clearance: novel targets for antidepressants
Depression is a major public health problem, especially in young people, because the few antidepressants that are available to treat children and adolescents are less effective than in adults. These antidepressants block uptake of serotonin in brain by the serotonin transporter. The goal of this project is to investigate the role of other, "non-traditional" transporters for serotonin in limiting the therapeutic effects of antidepressants, especially in children and adolescents, with the goal to guide development of more effective antidepressants to treat young people.

R01 DA029254 (France) 03/16 – 03/21
NIH/NIDA
Delay discounting: effects of drug dependence and withdrawal
The goal of this project is to examine the effects of chronic drug administration and its discontinuation (withdrawal) on delay discounting to determine how common drugs of abuse affect impulsivity.

Name: Julie Hensler
Project Role: Collaborator
Researcher Identifier: hensler (ERA Commons)
Nearest person month worked: 0.6 mos/yr
Contribution to Project: Dr. Hensler provides critical equipment for uptake studies, consultation for planning experiments and manuscripts, and other intellectual contributions as needed.

W81XWH-12-1-0506 (Gould, PI) 09/30/12 – 09/29/2015
Autism Idea Award AR11019 CDMRP/DOD
Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders
The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Hensler Other Active Support:
R01 NS065783 (Mazarati, PI) 05/01/2014-04/30/2018

Role: Co-Investigator 0.6 calendar
NIH sub through UCLA
Mechanisms of Co-Morbidity between Epilepsy and Depression
The present project is a continuation of our efforts to understand mechanisms of depression as a comorbidity of epilepsy. The goal of this project is to explore central noradrenergic dysfunction as a mechanism of depression linked to epilepsy in those subjects in which serotonergic transmission is not primarily compromised.

R01 MD009149 (Hensler, PI) 07/01/2014-06/30/2019
Role PI, 2.4 calendar
NIH
BDNF modulation of kynurenine pathway metabolism: neuroprotection during stress
Exposure to trauma or stress has been shown to be one of the main predisposing risk factors to psychiatric disorders. However, many individuals exposed to adversity maintain normal psychological functioning, and the factors underlying resistance to the deleterious effects of stress remain unknown. We propose that BDNF functions as a modulator of kynurenine metabolism in brain, and that it is through this mechanism that BDNF confers resilience to stress. The proposed studies include experiments at the cellular, systems and behavioral level utilizing two mouse models: BDNF heterozygous mice (BDNF+/- mice), which exhibit marked reductions in BDNF expression; transgenic mice carrying the met allele of the human bdnf gene (BDNFmet knock-in mice).

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Yes, changes affecting key personnel effort on this project have changed since the last reporting period. There was no research assistant following Corey Smolik's departure from 8/2015 – 3/2015 when Sergey Poplyaev was hired, and then overlap in research assistant effort with Marshall Edwards being hired from May – June until Sergey resigned. At the time of the original project end date, 9/29/2015 effort and salary coverage for Drs. Daws, Javors, Hensler and Koek ended, although Dr. Javors continues to perform measurements of D-22 for this project. Dr. Gould and Marshall Edwards each continue to work on the project with a 1.2 calendar months of effort from 9/29/2015 until the new project end date of 3/31/2016.

Partner Organizations
None to report

CONCLUSION/ IMPACT:

The findings from this project and its publications highlight the importance of serotonin neurotransmission in the brain in shaping adult social behavior, and how it is sensitive not only to selective serotonin reuptake inhibitors, but also to blockade of ancillary transporters of serotonin. Among these organic cation transporters (OCTs) and plasma membrane monoamine transporters (PMAT) are blocked by D-22 that reached the brain following acute systemic administraton and likely mediates its behavioral effects. These findings might also be extended to other fields within biomedical neuroscience such as schizophrenia or depression wherein impaired social behavior is prominent. Among inbred mouse strains, it appears as though there are differences in the capacity of 'uptake 2' transporters to remove 4-HT from extracellular fluid in the brain. This type of uptake is effectively blocked by D-22 in the low uM range, and it seems to differ in its blocking capacity from fluoxetine, which targets serotonin transporters. D-22 may have a short half-life in mice, but it tends to improve social behavior when administered systemically. The outcome of combined D-22 and fluoxetine blockade are of great interest, since we will determine if D-22 may be useful as a SSRI booster or as a complementary adjuvant to risperidone. D-22 appears to promote social behavior both with acute and sub-chronic treatment, indicating that OCT3 or other forms of uptake2 blockade may be beneficial for treatment of sociability impairments in autism and related disorders.

REFERENCES:

Baganz NL, Horton RE, Calderon AS, Owens WA, Munn JL, Watts LT, Koldzic-Zivanovic N, Jeske NA, Koek W, Toney GM, Daws LC. 2008. Organic cation transporter 3: Keeping the brake on extracellular serotonin in serotonin-transporter-deficient mice. Proc Natl Acad Sci U S A. 105:18976-81.

Carneiro AM, Airey DC, Thompson B, Zhu CB, Lu L, Chesler EJ, Erikson KM, Blakely RD. 2009. Functional coding variation in recombinant inbred mouse lines reveals multiple serotonin transporter-associated phenotypes. Proc Natl Acad Sci U S A.106:2047-52. PMID: 19179283.

Daws LC. 2009. Unfaithful neurotransmitter transporters: focus on serotonin uptake and implications for antidepressant efficacy. Pharmacol Ther. Jan;121(1):89-99.

Daws LC, Koek W, Mitchell NC. 2013. Revisiting serotonin reuptake inhibitors and the therapeutic potential of "uptake-2" in psychiatric disorders. ACS Chem Neurosci. 4:16-21.

Duan H, Wang J. 2010. Selective transport of monoamine neurotransmitters by human plasma membrane monoamine transporter and organic cation transporter 3. Journal of Pharmacology and Experimental Therapeutics. 335:743-53.

Gould GG, Hensler JG, Burke TF, Benno RH, Onaivi ES, Daws LC. (2011). Density and functionof central serotonin (5-HT) transporters, 5-HT1A and 5-HT2A receptors, and effects of their targeting on BTBR T+tf/J mouse social behavior. J Neurochem. 116:291-303.

Gould GG, Burke TF, Osorio MD, Smolik CM, Zhang WQ, Onaivi ES, Gu TT, DeSilva MN, Hensler JG. 2014. Enhanced novelty-induced corticosterone spike and upregulated serotonin 5-HT 1A and cannabinoid CB 1 receptors in adolescent BTBR mice. Psychoneuroendocrinology. 39:158-69.

Henry CA, Steingard R, Venter J, Guptill J, Halpern EF, Bauman M. 2006. Treatment outcome and outcome associations in children with pervasive developmental disorders treated with selective serotonin reuptake inhibitors: a chart review. J Child Adolesc Psychopharmacol. 16:187-95.

Henry CA, Shervin D, Neumeyer A, Steingard R, Spybrook J, Choueiri R, Bauman M. 2009. Retrial of selective serotonin reuptake inhibitors in children with pervasive developmental disorders: a retrospective chart review. J Child Adolesc Psychopharmacol. 19:111-7. PMID: 19364289.

Hill JE, Makky K, Shrestha L, Hillard CJ, Gasser PJ. 2011. Natural and synthetic corticosteroids inhibit uptake 2-mediated transport in CNS neurons. Physiol Behav. 104:306-11.

Horton RE, Apple DM, Owens WA, Baganz NL, Cano S, Mitchell NC, Vitela M, Gould GG, Koek W, Daws LC. 2013. Decynium-22 enhances SSRI-induced antidepressant-like effects in mice: uncovering novel targets to treat depression. The Journal of Neuroscience. 33:10534-43.

Lee N, Duan H, Hebert MF, Liang CJ, Rice KM, Wang J. 2014. Taste of a pill: organic cation transporter-3 (OCT3) mediates metformin accumulation and secretion in salivary glands. J Biol Chem. 289(39):27055-64.

McPheeters ML, Warren Z, Sathe N, Bruzek JL, Krishnaswami S, Jerome RN, Veenstra-Vanderweele J. 2011. A systematic review of medical treatments for children with autism spectrum disorders. Pediatrics. 127:e1312-21.

Perdan-Pirkmajer K, Pirkmajer S, Černe K, Kržan M. 2012. Molecular and kinetic characterization of histamine transport into adult rat cultured astrocytes. Neurochem Int. 6:415-22.

Pobbe RL, Defensor EB, Pearson BL, Bolivar VJ, Blanchard DC, Blanchard RJ. 2011. General and social anxiety in the BTBR T+ tf/J mouse strain. Behav Brain Res. 216:446-51.

Sakurai E, Sakurai E, Oreland L, Nishiyama S, Kato M, Watanabe T, Yanai K. 2006. Evidence for the presence of histamine uptake into the synaptosomes of rat brain. Pharmacology. 78:72-80.

Soorya L, Kiarashi J, Hollander E. 2008. Psychopharmacologic interventions for repetitive behaviors in autism spectrum disorders. Child and adolescent psychiatric clinics of North America. 17:753-71.

West L, Waldrop J, Brunssen S. 2009a. Pharmacologic treatment for the core deficits and associated symptoms of autism in children. J Pediatr Health Care. 23:75-89.

West L, Brunssen SH, Waldrop J. 2009b. Review of the evidence for treatment of children with autism with selective serotonin reuptake inhibitors. J Spec Pediatr Nurs. 14:183-91.

Williams K, Brignell A, Randall M, Silove N, Hazell P. 2013. Selective serotonin reuptake inhibitors (SSRIs) for autism spectrum disorders (ASD). Cochrane Database Syst Rev. 8:CD004677.

Yoshikawa T, Naganuma F, Iida T, Nakamura T, Harada R, Mohsen AS, Kasajima A, Sasano H, Yanai K. 2013. Molecular mechanism of histamine clearance by primary human astrocytes. Glia. 61:905-16.

Zhang WQ, Smolik CM, Barba-Escobedo PA, Gamez M, Sanchez JJ, Javors MA, Daws LC, Gould GG. 2015. Acute dietary tryptophan manipulation differentially alters social behavior, brain serotonin and plasma corticosterone in three inbred mouse strains. Neuropharmacology. 290:1-8.

SUPPORTING DATA: N/A since figures and/or tables are included in the text.

APPENDICES: Key manuscripts reflecting findings and project effort that have been published.

Psychoneuroendocrinology (2014) 39, 158—169

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Enhanced novelty-induced corticosterone spike and upregulated serotonin 5-HT_{1A} and cannabinoid CB_1 receptors in adolescent BTBR mice

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KEYWORDS
129S1/SvImJ;
Adolescent;
BTBR;
C57BL;
Cannabinoid;
GTPγS autoradiography;
Hippocampus;
HPA feedback;
Serotonin;
Social behavior

Summary Hypothalamic pituitary adrenal (HPA) axis responses to change and social challenges during adolescence can influence mental health and behavior into adulthood. To examine how HPA tone in adolescence may contribute to psychopathology, we challenged male adolescent (5 weeks) and adult (16 weeks) BTBR T$^+$tf/J (BTBR) and 129S1/SvImJ (129S) mice with novelty in sociability tests. In prior studies these strains had exaggerated or altered HPA stress responses and low sociability relative to C57BL/6J mice in adulthood. In adolescence these strains already exhibited similar or worse sociability deficits than adults or age-matched C57 mice. Yet BTBR adolescents were less hyperactive and buried fewer marbles than adults. Novelty-induced corticosterone (CORT) spikes in adolescent BTBR were double adult levels, and higher than 129S or C57 mice at either age. Due to their established role in HPA feedback, we hypothesized that hippocampal Gαi/o-coupled serotonin 5-HT_{1A} and cannabioid CB_1 receptor function might be upregulated in BTBR mice. Adolescent BTBR mice had higher hippocampal 5-HT_{1A} density as measured by [^{3}H] 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) binding than C57 mice, and adult BTBR 8-OH-DPAT-stimulated GTPγS binding was higher than in either C57 or 129S mice in this region. Further, BTBR hippocampal CB_1 density measured by [^{3}H]CP55,940 binding was 15—20% higher than in C57.

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CP55,940-stimulated GTPγS binding in adult BTBR dentate gyrus was 30% higher then 129S ($p < 0.05$), but was not a product of greater neuronal or cell density defined by NeuN and DAPI staining. Hence hyperactive HPA responsiveness during adolescence may underlie 5-HT_{1A} and CB_1 receptor up-regulation and behavioral phenotype of BTBR mice.

1. Introduction

Exposure to severe stressors during adolescence can persistently alter hypothalamic pituitary adrenal (HPA) axis response, perception, cognition and mood into adulthood (McCormick and Mathews, 2007; Stevens et al., 2009). For example, unstable childhood home environments are associated with exaggerated HPA responses to stress, particularly among males (Hackman et al., 2012; Brenner et al., 2013). However, teenage boys with autism, depression or other psychiatric disorders often exhibit exaggerated HPA responses (e.g. higher cortisol peaks) following social or novel stimuli than their 'normotypic' peers, paralleling individuals severely stressed in youth, even without a prior history of severe stress exposure (Lopez-Duran et al., 2009; Corbett et al., 2010; Spratt et al., 2012; Schupp et al., 2013). This low HPA axis resilience can have profound long-term impacts on social behavior, and may also increase the risk of suicide (Sher, 2006; Sunnqvist et al., 2008; Garlow et al., 2008).

Paralleling this, in male BTBR T+tf/J (BTBR) and 129S1/SvImJ (129S) mice exhibiting low social interaction (Gould et al., 2011; 2012), stressors induce exaggerated peak corticosterone (CORT) levels. For example, stressed BTBR released more CORT, yet BTBR have higher hippocampal glucocorticoid receptor (GR) mRNA levels than C57BL/6 mice (Benno et al., 2009; Silverman et al., 2010). 129S mice also had higher post-stress peak CORT, but had lower hippocampal GR mRNA than C57BL/6 mice (Camp et al., 2012). All mice in these studies were adults. Since murine stress reactivity appears to be exaggerated prior to puberty (Romeo et al., 2013), yet adolescent and adult hippocampal GR expression are similar (Pryce, 2008), adolescent HPA axis tone was of great interest in these strains. Hence we compared adolescent and adult CORT responses to mild stressors, specifically novel social interaction and novel object exposure.

Corticosteroids bind to hippocampal GRs, the activation of which promotes serotonin transmission that in turn attenuates CORT release (Lanfumey et al., 2008; Pompili et al., 2010). CORT binding to GR also increases hippocampal endocannabinoid levels, and this likewise suppresses CORT release (Cota, 2008; McLaughlin et al., 2009; Atsak et al., 2012). This HPA axis suppression is likely mediated via inhibitory metabotrophic Gαi/o coupled serotonin 5-HT_{1A} and CB_1 receptors. When direct HPA axis feedback by circulating CORT via GRs is impaired, 5-HT_{1A} and CB_1 receptors may be up-regulated in compensation (Lanfumey et al., 2008; Pompili et al., 2010; Hensler et al., 2010). We hypothesized that this type of compensation may occur to a greater extent during adolescence, particularly if the HPA stress-response is exaggerated.

We have found that in adult BTBR mice hippocampal 5-HT_{1A} receptor function was enhanced relative to C57BL/10 mice (Gould et al., 2011). We hypothesized that exaggerated stress-evoked CORT release during adolescence could underlie this 5-HT_{1A} up-regulation, and may likewise up-regulate hippocampal CB_1 receptors. Since higher binding could stem from increases either in receptor expression or neuronal density, we compared hippocampal neuronal cell density in adults of each strain. Further, receptor up-regulation might be more pronounced in adolescent mice with HPA axis hyperactivity. Thus, we compared adolescent to adult 5-HT_{1A} and CB_1 receptor densities and agonist-stimulated G-protein coupling in these strains.

2. Methods

2.1. Mouse subjects

BTBR T+tf/J, 129S1/SvImJ, C57BL/10J and C57BL/6J colony founders were from Jackson Laboratory (Bar Harbor, ME, USA). The mice were second or greater generation offspring bred in the laboratory animal facilities at William Paterson University, Wayne, NJ for quantitative autoradiography, or at The University of Texas Health Science Center at San Antonio (UTHSCSA), San Antonio, TX for autoradiography, immunostaining, behavior and corticosterone measures. Mice at both facilities were maintained at 20—24 °C on 12:12 light dark cycles, with lights on at 0700 h, and ad libitum access to food and water in cages lined with wood chip bedding (at UTHSCSA) or shaved wood bedding (at William Patterson University) that was changed bi-weekly. Mice were weaned at around postnatal day 21 and were housed in same-sex littermate groups of 2—5 per cage. All procedures involving mice were approved by Institutional Animal Care and Use Committees, and were consistent with current NIH guidelines.

2.2. Novelty exposure and plasma CORT levels

Mice used for novelty exposures and corticosterone (CORT) measures were 5 or 16 week-old male C57BL/6, 129S and BTBR. Prior to trunk blood collection, half of the mice were subjected to three-chamber sociability tests (40 min), immediately followed by marble burying (30 min), as in Gould et al. (2011, 2012). Briefly, each mouse subject was individually placed in a novel three-chamber arena for sociability testing. First subjects explored their arena for 20 min of pre-conditioning, then an unfamiliar object (wire cup) and 'stranger 1' a 129S male mouse (4—5 weeks old, in wire cup) was introduced at either end for 10 min, and finally a second novel 'stranger 2' mouse under a wire cup was introduced for 10 min, replacing the empty cage. Self-grooming was only scored during the sociability test and was not scored in an independent task. Afterward each subject mouse was transferred for 30 min to a 51 cm × 28 cm × 23 cm box filled with

10 cm of wood-chip bedding topped with 15 blue marbles in a grid pattern. The untested group of mice remained in their home cages until sacrifice. All mice were humanely sacrificed by decapitation between 1500 and 1650 h CST, and trunk blood was collected into tubes containing 25 μl of 20 mM ethylenediaminetetraacetic acid (Sigma, St Louis, MO).

Mouse blood was centrifuged at 4 °C for 10 min at 2600 rpm, and serum collected and frozen at −80 °C. Plasma CORT levels were measured colorometrically on a plate reader (Molecular Devices, Sunnyvale, CA) after using an enzyme immunoassay kit (#ADI-900-097, Enzo, Plymouth Meeting, PA), following the manufacturer's small sample volume protocol.

2.3. Brain tissue preparation

Mice used for quantitative autoradiography and immunostaining were 5 and/or 16 week old naïve males. The mice were sacrificed by decapitation and their brains were frozen on powdered dry ice and stored at −80 °C. Coronal sections (20 μm) were collected from 0.90 to 0.60 mm and −1.80 to −2.00 mm relative to Bregma on a cryostat (Leica, Buffalo Grove, IL), and were thaw mounted onto chilled gelatin coated microscope slides. The sections were desiccated for 12—24 h under vacuum at 4 °C and then stored at −80 °C until use in autoradiography or immunostaining.

2.4. Quantitative autoradiography

Binding assays with [^{3}H] 8-OH-DPAT (2 nM) for serotonin 5-HT_{1A}, with 1 μM WAY 100,635 (Tocris, Ellisville, MO) to define non-specific binding, and [^{3}H] CP55,940 (5 nM) for cannabinoid CB_1 receptors, with 200 μM WIN,55-212-2 (Ascent Scientific, Princeton NJ) for non-specific binding, were performed on brain sections as per Gould et al. (2012). [^{35}S] GTPγS binding in the absence or presence of 1 μM of agonists 8-OH-DPAT or CP 55,940 was performed as described in Gould et al. (2011, 2012). Radioligands were from Perkin-Elmer (Boston, MA), and Kodak Biomak MR film (ThermoFisher, Waltham, MA) was used for all experiments.

Digital images were captured on a camera (1612M, Scion Corp., Frederick, MD) with a 60 mM lens onto a Macintosh (OS 10), with Image J software (NIH, Bethesda, MD) for density measures. Gray scale units were converted to fmol/mg protein or nCi/mg using calibration standards (American Radiolabeled Chem., St. Louis, MO), as per Gould et al. (2011). Brain regions measured included the hippocampus CA1, CA3 and dentate gyrus regions, frontal and parietal cortex, caudate putamen, nucleus acumbens (CB_1 only), amygdala and hypothalamus. The amygdala and hypothalamus were not measured for GTPγS due to high basal binding.

2.5. Immunohistochemical labeling

Brain sections on slides were thawed, rinsed with Dulbecco's phosphate buffered saline (DPBS, Life Technologies, Grand Island, NY) and fixed with 4% paraformaldehyde at 4 °C for 1 h. They were rinsed in DPBS 3 times for 5 min each, and permeablized in 0.5% triton X-100 in DPBS at 26 °C for 1 h. The tissue was blocked for 1 h at 26 °C, anti-NeuN (rabbit polyclonal, Millipore, Billerica, MA) 1:500 was added for an overnight incubation at 4 °C. The sections were washed 3 times with DPBS for 5 min each, and Alexa Fluor 488 goat anti-rabbit (Millipore) 1:500 secondary antibody was added before incubating for 2 h at 26 °C. After a final round of 3, 5 min DPBS washes, sections were cover slipped with vectashield mounting medium containing 4′,6-diamino-2-phenylindole dihydrochloride (DAPI, Vector Labs, Burlingame, CA). Sections were digitally captured on an Eclipse TE-2000-E (T-HUBC) microscope with a digital camera (Model: DXM1200C, Nikon, Melville, NY). Staining intensity in the dentate gyrus of hippocampus was measured using Nikon NIS-Elements AR 3.0 software.

2.6. Statistical analysis and sample sizes

Three-way (strain × age × treatment) analysis of variance (ANOVA) was used to compare mean CORT levels, with Fisher's least significant difference (LSD) post hoc tests performed when significant main effects occurred. Repeated measures ANOVA was used to compare time in chamber and sniffing time in sociability tests, significant effects were further resolved by post hoc ANOVA and/or *t*-test comparisons. Two-way ANOVA was used to compare mean chamber entries, grooming times, and marbles buried, with Fisher's LSD post hoc tests performed to resolve significant effects. There were 6—9 mice per strain/age/treatment group. For autoradiography two-way (strain × age) multivariate (several brain regions measured) ANOVA was used to compare mean binding density for several different brain regions, with Newman Keuls post hoc tests performed, there were 8 mice per group. ANOVA was used to compare immunostaining intensity in the dentate gyrus, there were 7—8 mice per strain. All analyses were performed using Statistica software (StatSoft, Tulsa, OK).

3. Results

3.1. Effects of strain, age and behavior tests on plasma CORT

Corticosterone levels increased significantly after 70 min of novelty exposure in behavior tests ($F_{1,66} = 177$, $p < 0.0001$), and differed in magnitude among mouse strains ($F_{2,66} = 24$, $p < 0.0001$) and ages ($F_{1,66} = 17$, $p < 0.0001$), with significant interactions ($p < 0.017$). The post-novelty increase in CORT was two-fold greater in 5 week-old BTBR mice than other strains, and was also greater than in adult BTBR mice ($p < 0.0001$) (Fig. 1). Baseline CORT levels tended to be higher in adult BTBR than C57BL/6, and this difference was nearly significant ($p = 0.06$).

3.2. Social and repetitive behaviors in adolescent and adult male mice

In global analysis of the three-chamber sociability tests, there was a significant interaction between mouse strain and age for time spent in either end chamber of the test arena ($F_{2,30} = 3.23$, $p < 0.05$). In the social interaction phase, C57 adults spent significantly more time by the stranger mouse vs. novel object ($t = -3.8$, $p < 0.05$), while C57 adolescents also tended to, this trend was not significant

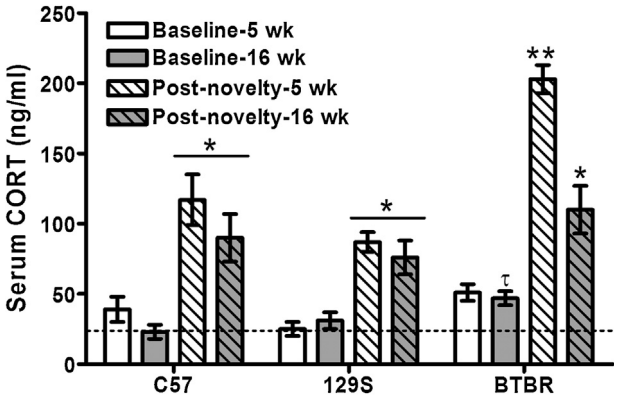

Figure 1 Novelty exposure raised plasma CORT levels the most in adolescent BTBR mice. CORT levels increased above mean baseline levels in adolescent (5 week) and adult (16 week) male mice of C57BL/6, 129S1/SvImJ and BTBR strains after 70 min of novelty exposure in behavior tests (*$p < 0.05$). However, CORT levels in novelty-exposed 5 week-old BTBR mice were far higher than all of the other strains and ages (**$p < 0.0001$). Baseline CORT levels tended to be significantly higher in BTBR than in C57BL/6 adults (τ $p = 0.06$). The dashed horizontal line illustrates the mean CORT level found in adult C57BL/6 mice to facilitate visual reference.

($t = -2.14$, $p = 0.09$). Five week-old BTBR mice spent less time in chambers with stranger mice and more time in chambers with novel objects ($t = 3.2$, $p < 0.02$), and they differed in comparison to BTBR adults with respect to this measure ($F_{1,10} = 4.95$, $p < 0.05$, Fisher's LSD $p < 0.05$, Fig. 2a). In the second phase of the test, only C57BL/6 adult mice showed a significant preference for social novelty based on time spent in the chamber with the new stranger mouse ($p < 0.05$, Fig. 2b).

A global repeated measures analysis of social sniff time during sociability tests revealed significant differences between ages ($F_{1,30} = 15$, $p < 0.001$), test phase ($F_{1,30} = 25$, $p < 0.0001$), with significant interactions among all main effects. With the finding of a significant age effect in the repeated measures ANOVA ($F_{1,30} = 9.45$, $p < 0.01$), in post hoc ANOVAs performed on the social interaction phase, there was a significant effect of age for the amount of time spent sniffing empty cages ($F_{1,30} = 15$, $p < 0.001$), and an interaction between strain and age ($F_{2,30} = 3.9$, $p < 0.32$). Specifically BTBR adolescents spent more time sniffing the empty cages than all other groups except 5 week-old 129S mice. There was no significant difference in social sniff of stranger mice between strain ($p = 0.4$), or age ($p = 0.7$), as shown in Fig. 2c. However only C57 adults and adolescents had a significant preference for stranger mice ($t > 3.4$, $p < 0.05$). In the social novelty phase, there was a significant effect of strain ($F_{1,30} = 15$, $p < 0.0001$) and age ($F_{2,30} = 0.001$), with interactions among factors ($F_{2,30} = 12$, $p < 0.0001$). This was due to an increased amount of sniffing by 129S adolescents of either stranger relative to all other strains ($F_{2,30} > 5$, $p < 0.05$, Fisher's LSD $p < 0.05$), and more sniffing of the new strangers in comparison to 129S adults ($F_{1,30} = 22$, $p < 0.0001$, Fisher's LSD $p < 0.05$), shown in Fig. 2d.

During social interaction tests, BTBR adults were hyperactive, making more chamber entries than other groups ($F_{2,30} = 4.7$, $p < 0.02$; Fisher's $p < 0.05$), yet their self-grooming duration across both sociability test phases combined was similar to other groups ($F_{2,30} = 0.87$, $p = 0.42$, Fig. 2e). In contrast, 129S mice spent significantly less time self-grooming than C57BL/6 mice during the sociability tests ($F_{2,30} = 5.1$, $p < 0.01$; $p < 0.05$, Fig. 2e), while C57BL/6 and BTBR self-grooming was similar. Immediately after sociability tests, adult BTBR and 5-week 129S mice buried more marbles than C57BL/6 mice ($F_{2,30} = 13$, $p < 0.001$, Fisher's LSD $p < 0.05$, Fig. 2f).

3.3. Serotonin 5-HT$_{1A}$ receptors

There were significant differences in [^{3}H] 8-OH-DPAT binding density among strains (Wilks' $\lambda_{16,70} = 0.17$, $p < 0.0001$) and ages ($\lambda_{8,35} = 0.32$, $p < 0.0001$), with interactions among these factors ($\lambda_{16,70} = 0.51$, $p = 0.05$) in the hippocampus and in other brain areas. Among 16 week-old mice, 129S [^{3}H] 8-OH-DPAT binding was higher in the CA1 of hippocampus (Fig. 3b) and cingulate cortex (by roughly 30%) than either BTBR or C57BL/10 ($F_{2,42} > 8$, $p < 0.01$; Newman–Keuls $p < 0.05$). Further, 5-week old 129S and BTBR mice had higher [^{3}H] 8-OH-DPAT binding in the CA1 of hippocampus than C57BL/10 at either age ($F_{2,42} = 21$, $p < 0.001$; $p < 0.01$, Fig. 3b). Five-week old BTBR mice also had higher [^{3}H] 8-OH-DPAT binding in the CA3 of hippocampus (Fig. 3c, $F_{2,42} = 7.1$, $p < 0.002$; $p < 0.05$). Also, 16-week old BTBR mice had lower [^{3}H] 8-OH-DPAT binding than 5-week olds in the basolateral amygdala (108 ± 14 vs. $\approx$165 fmol/mg protein) and ventromedial hypothalamus (129 ± 11 vs. $\approx$200 fmol/mg protein) ($F_{1,42}$ or $F_{2,42} > 3.15$, $p < 0.05$). Non-specific was 8–10% of total binding.

8-OH-DPAT-stimulated [^{35}S] GTPγS binding tended to differ among strains ($\lambda_{8,78} = 0.11$, $p = 0.067$), but not among ages ($\lambda_{5,38} = 0.87$, $p = 0.38$), with no interaction ($\lambda_{10,76} = 0.84$, $p > 0.7$). Specifically, agonist-stimulated 5-HT$_{1A}$ binding in 16 week-old BTBR mice was higher in all hippocampal regions measured ($F_{2,42} > 3$, $p \leq 0.05$), as shown in the plots on the right side in Fig. 3a–c. Basal binding ranged from a mean of 249 ± 14 to 435 ± 31 nCi/mg in the brain regions measured, and non specific binding was roughly 40% of basal and 10% of stimulated binding.

Taken together, the age-dependent density and functional relationship between 5-HT$_{1A}$ and 8-OH-DPAT stimulated GTPγS binding in the hippocampus of BTBR mice (Fig. 3a–c) is of particular interest, given this receptor's proposed modulatory role in HPA axis feedback. Specifically [^{3}H] 8-OH-DPAT binding density in 5-week old mice was higher in BTBR than in C57BL/10 mice in the CA1 ($p < 0.01$) and CA3 ($p < 0.005$) regions of the hippocampus, yet at 16 weeks of age, [^{35}S]GTPγS binding was significantly higher in BTBR mice than in C57BL/10 mice in the CA1 ($p = 0.05$) and dentate gyrus ($p < 0.05$) regions of the hippocampus.

3.4. Cannabinoid CB$_1$ receptors

There were differences in [^{3}H] CP55,940 binding density among strains ($\lambda_{18,68} = 0.1$, $p < 0.001$), specifically C57BL/10 had lower density than BTBR and/or 129S mice in dentate gyrus, CA1, CA3 of hippocampus and in the parietal cortex (175 vs. >202 fmol/mg protein) at 5 weeks, and in the dentate gyrus and hippocampal CA3 at 16 weeks of age

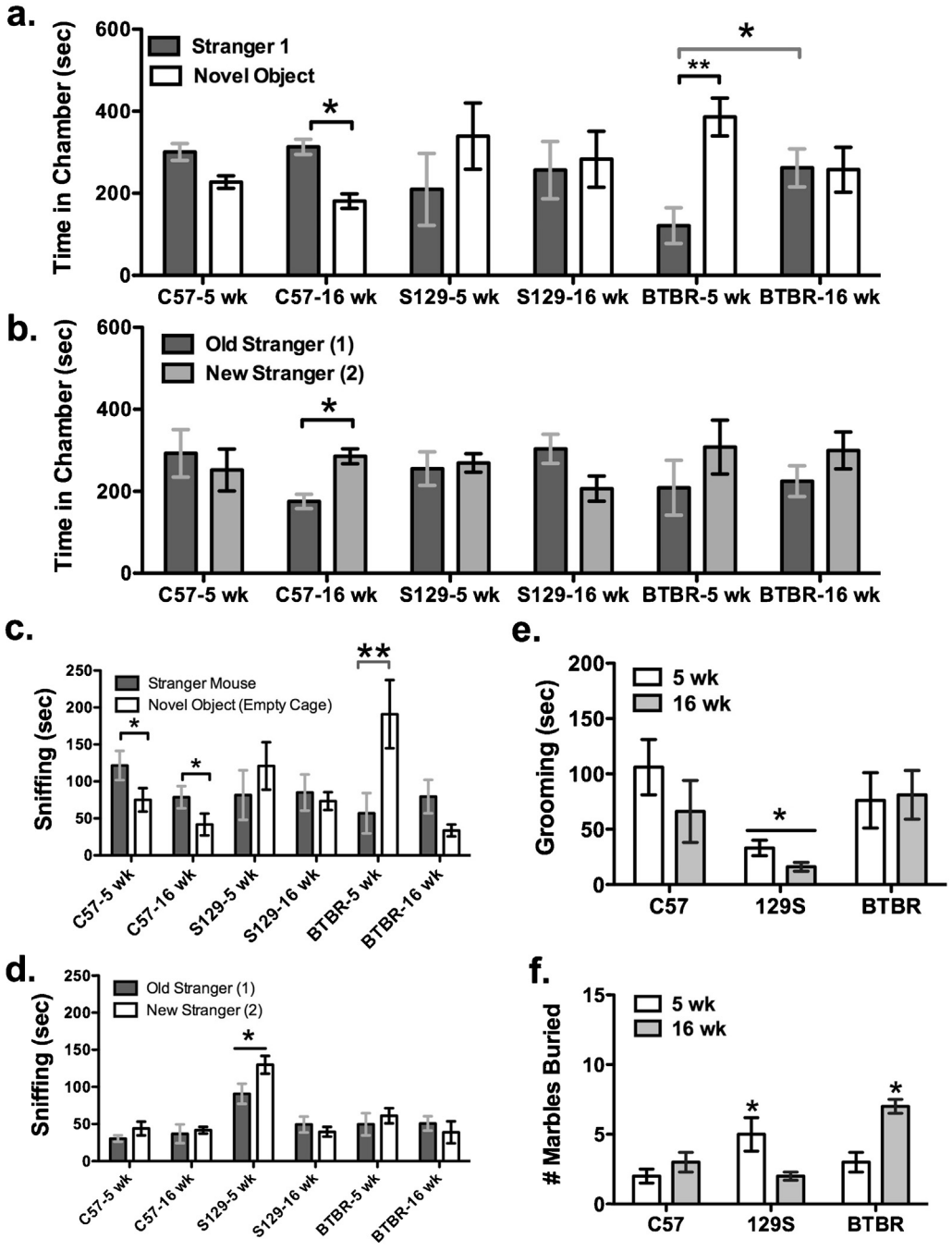

Figure 2 Behavior of adolescent and adult BTBR mice in sociability and repetitive tests. (a) A significant preference for social interaction was only found in C57 adults (*$p < 0.05$). Adolescent BTBR mice spent more time in chambers with novel objects than with stranger mice in the social interaction test (**$p < 0.001$), in contrast to adults (*$p < 0.01$). (b) Only adult C57 mice spent significantly more time in chambers with new strangers than with the 'old' original ones (*$p < 0.05$). (c) Both C57 adolescent and adult mice spent more time sniffing stranger mice than novel objects (*$p < 0.05$), however BTBR adolescents spent significantly more time sniffing novel objects than all other mice (**$p < 0.001$). (d) Time spent sniffing new and old stranger mice during the social novelty test was greatest in 129S adolescents (*$p < 0.05$). (e) Self-grooming among C57 and BTBR mice was comparable, but was lower in 129S mice during sociability testing (*$p < 0.05$). (f) BTBR adults and 129S adolescents buried more marbles over 30 min after sociability testing than C57BL/6 mice (*$p < 0.05$).

($F_{2,42} > 3$, $p < 0.05$, Fig. 4a–c, left panels). While a significant age effect was detected ($\lambda_{9,34} = 0.29$, $p < 0.001$), post hoc ANOVA failed to reveal age differences in the regions measured ($F_{1,42} < 2$, $p > 0.15$), and there were no interactions ($\lambda_{18,68} = 0.5$, $p = 0.19$). Non-specific binding was 40–50% of total.

CB_1 receptor CP55,940-stimulated [^{35}S] GTPγS binding differed among strains ($\lambda_{10,76} = 0.55$, $p < 0.01$) and ages ($\lambda_{5,38} = 0.65$, $p < 0.01$) without interaction ($\lambda_{10,76} = 0.67$, $p = 0.1$). In all strains, CP55,940-stimulated binding increased with age in the hippocampus, as shown in the right panels of Fig. 4, and also in the cingulate cortex (18–40%

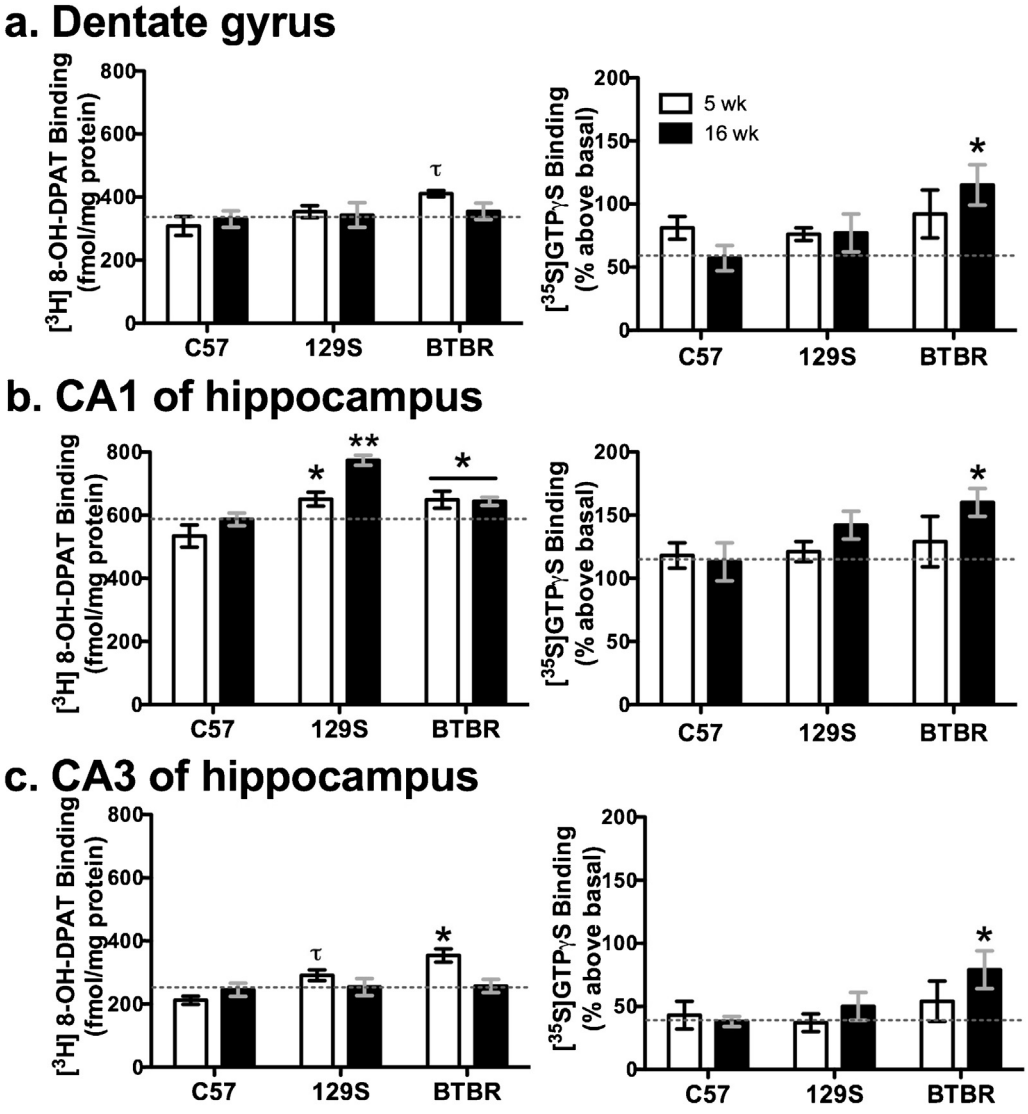

Figure 3 Specific [^{3}H] 8-OH-DPAT binding and 8-OH DPAT-stimulated [^{35}S] GTPγS binding to serotonin 5-HT$_{1A}$ receptors in the hippocampus of adolescent and adult mice. In the left-side panels [^{3}H] 8-OH-DPAT binding was higher in 5-week old BTBR and/or 129S mice in (b) the CA1 and (c) CA3 of hippocampus than age-matched C57 mice (*$p < 0.05$; **$p < 0.01$; τ $p = 0.067$). In (a) the dentate gyrus, there was also a non-significant trend toward BTBR mice at 5 weeks having greater [^{3}H] 8-OH-DPAT binding (τ $p = 0.085$). In the right-side panels 8-OH-DPAT stimulated [^{35}S] GTPγS binding was enhanced in adult BTBR mice in (a) dentate gyrus, (b) CA1 and (c) CA3 of hippocampus as compared to adult C57 (*$p \leq 0.05$). Dashed horizontal lines are a reference marker for mean density in adult C57 mice. Thus, in 5-week old BTBR mice hippocampal 5-HT$_{1A}$ density was high, whereas in adults 5-HT$_{1A}$ function was enhanced.

greater in adults). In 16 week-old BTBR mice CP55,940-stimulated [^{35}S] GTPγS binding was higher in dentate gyrus than in other strains ($F_{2,42} = 3.7$, $p < 0.05$, Fig. 4a, right). We also observed in the hippocampal CA1 and CA3 of 5 week-old 129S mice that CP55,940-stimulated [^{35}S] GTPγS binding was lower ($p < 0.05$) than in C57BL/10 mice of the same age. This corresponded with modest increases in 129S CB$_1$ receptor density in the 5-week old hippocampus, as measured by [^{3}H] CP55,940.

3.5. C57BL/10 vs. C57BL/6 binding comparison

Because C57BL/6 mice are more commonly used than C57BL/10, we compared [^{3}H] 8-OH-DPAT, [^{3}H] CP55,940, and agonist stimulated [^{35}S] GTPγS binding density in them. We did not observe any significant differences in 5-HT$_{1A}$ ($\lambda_{8,7} = 0.28$, $p = 0.15$) or CB$_1$ ($\lambda_{9,6} = 0.44$, $p = 0.61$) binding site density among these strains. Further, agonist-stimulated [^{35}S] GTPγS binding did not reveal any real differences among C57BL/6 and C57BL/10 adults ($\lambda_{5,2} = 0.05$, $p = 0.12$).

3.6. Immunostaining in dentate gyrus

There were no differences in stain intensity for DAPI ($F_{2,19} = 2.8$, $p = 0.09$) or NeuN ($F_{2,19} = 1.0$, $p = 0.38$) in the dentate gyrus among adult male 129S, BTBR or C57BL/6 mice. NeuN stain intensity ranged from a mean ± S.E.M. of 43 ± 9

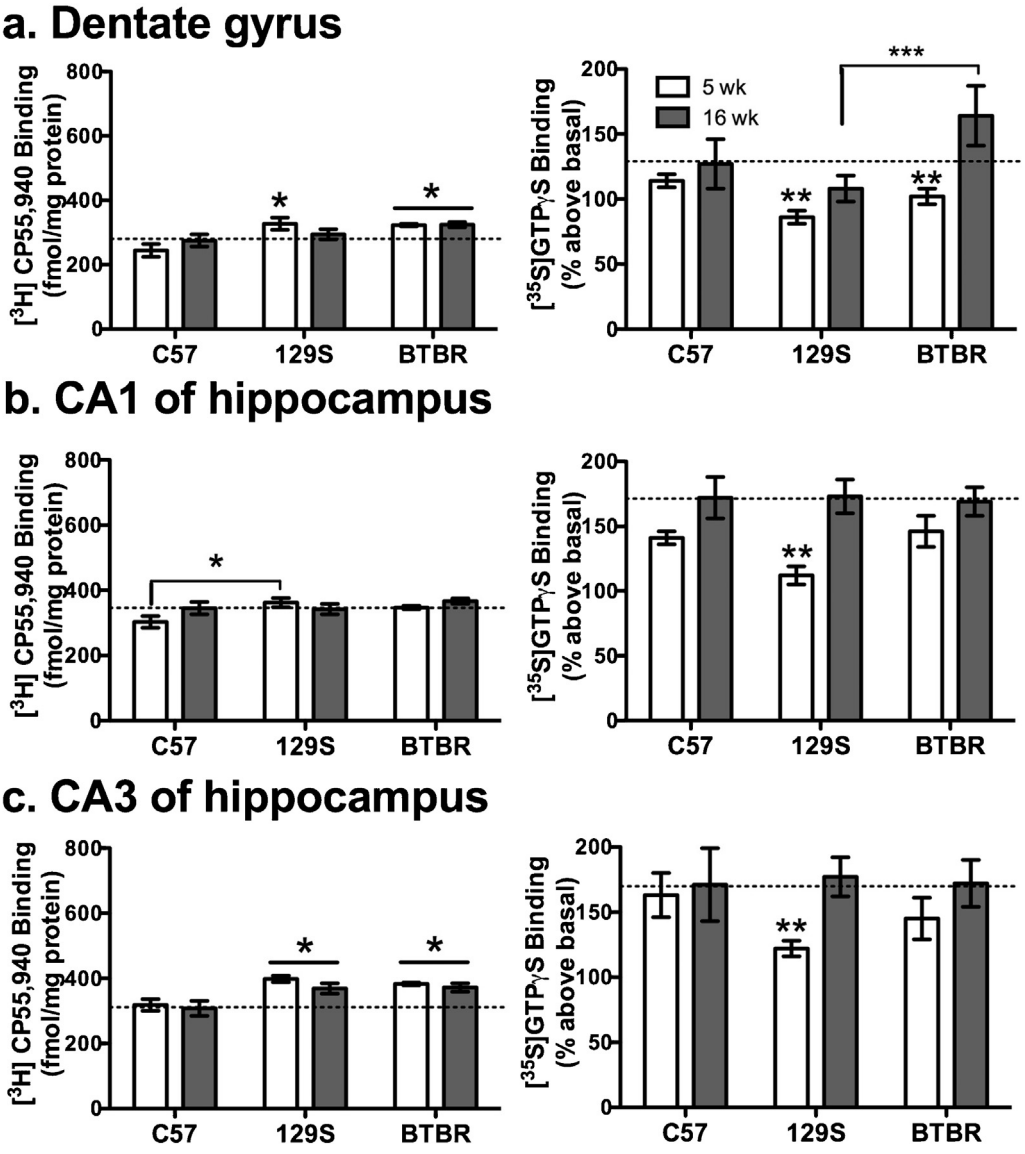

Figure 4 Specific [^{3}H] CP55,940 binding and CP55940-stimulated [^{35}S] GTPγS binding to hippocampal cannabinoid CB_1 receptors in adolescent and adult mice. As shown in the left-side panels [^{3}H] CP55,940 binding was greater in the (a) dentate gyrus and (c) CA3 of BTBR vs. C57 mice (*$p < 0.05$). Adolescent 129S mice had significantly higher [^{3}H] CP55,940 binding in the (a) dentate gyrus, (b) CA1 and (c) CA3 than adolescent C57 mice (*$p < 0.05$). On the other hand, CP55,940 stimulated [^{35}S] GTPγS binding generally increased with age, as shown in the panels to the right. However, hippocampal CP55,940 stimulated [^{35}S] GTPγS binding was consistently lower in 129S adolescents (**$p < 0.05$, a–c). Also agonist stimulated GTPγS binding was higher in adult BTBR than in 129S mice (***$p < 0.05$, a). Dashed horizontal lines are a reference marker for mean density in adult C57 mice. Thus CB1 receptor coupling capacity is reduced, despite elevated CB1 receptor density in the adolescent 129S hippocampus.

to 64 ± 11 optical density units, while DAPI ranged from 72 ± 7 to 92 ± 5 density units. Representative images are shown in Fig. 5.

4. Discussion

Herein we provide evidence of greater novelty-induced increases in serum CORT in adolescent male BTBR mice, accompanied by greater hippocampal serotonin 5-HT_{1A} and cannabinoid CB_1 receptor density relative to C57 mice. In BTBR adults, agonist-stimulated GTPγS binding to hippocampal 5-HT_{1A} receptors was augmented, but this was not accompanied by higher receptor, cellular or neuronal density. Enhanced hippocampal 5-HT_{1A} Gαi/o-coupled receptor activation capacity could facilitate inhibition of CORT release, and as such it may be a key compensatory response in adult BTBR HPA axis feedback. While hippocampal CB1 receptor density was greater in BTBR and 129S mice, CP55,940 stimulated GTPγS binding in 129S was no higher than, and in adolescents it was reduced relative to C57 mice. These factors may contribute to the age-dependent patterns in sociability deficits and hyperactivity we observed in BTBR and 129S males.

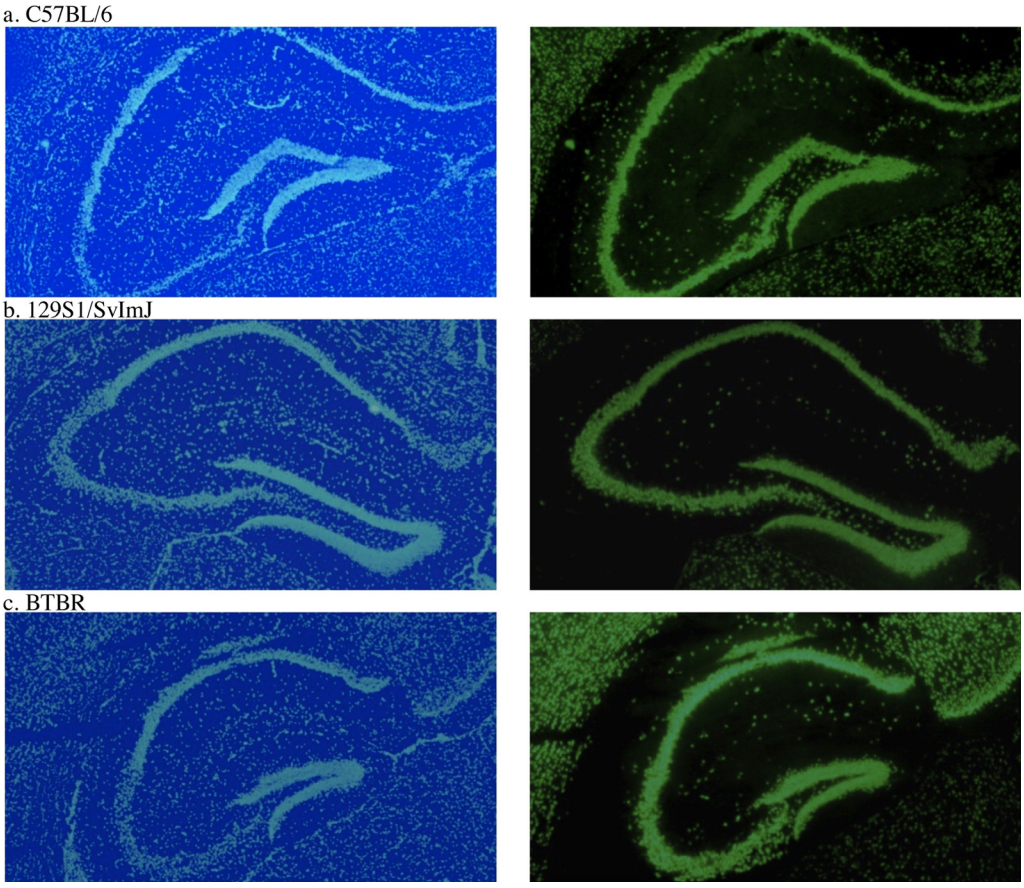

Figure 5 DAPI nuclear (left) and NeuN neuronal (right) staining in the adult mouse hippocampus. (a) C57BL/6, (b) 129S1/SvImJ and (c) BTBR mouse hippocampi had similar cell (in light blue) and neuron (in light green) density in the dentate gyrus.

4.1. Social behavior and CORT level comparisons across age and strains

A preference for chambers with novel objects over stranger mice — that might arguably be interpreted as social avoidance — was evident in adolescent male BTBR relative to C57BL/6 mice. This behavioral pattern was pronounced, even as compared to BTBR adults that generally lack preference for social interaction (Moy et al., 2007; Silverman et al., 2010). It also corresponded with higher levels of CORT in plasma after behavior testing. No similar age-dependent sociability patterns occurred in either C57 or 129S mice. Further, adult, but not adolescent BTBR mice entered the chambers in the sociability testing arena more frequently than other strains, consistent with earlier findings of hyperactivity in this strain (Silverman et al., 2010).

Based on prior studies, we predicted that CORT spikes in response to novel stimuli, at least in the adolescent C57BL/6 males, might be exaggerated compared to those in adults (Romeo et al., 2013), and that even higher levels might be found in BTBR mice (Benno et al., 2009). Indeed, we saw an exaggerated CORT response to novelty in adolescent BTBR after 70 min of sociability testing and marble burying. Our CORT level measures were also consistent with earlier reports of higher baseline and hyperactive HPA axis responsiveness in BTBR relative to C57BL/6 mice (Benno et al., 2009; Silverman et al., 2010). In contrast, CORT levels in 129S mice were no higher than C57BL/6 following novelty exposure, although restraint stress produced greater initial increases in them in a prior study (Camp et al., 2012). Since we did not measure CORT levels at earlier time points (e.g. at 30 min intervals) during our behavior tests, we suspect that we did not capture peak CORT levels induced by handling and novelty exposure. However, higher peak levels and/or extended release of CORT was nonetheless evident in BTBR adolescents after novelty exposure under our testing paradigm, and we also saw higher CORT levels in all strains and ages after behavior testing as compared to baseline values.

However, elevated CORT levels, either at baseline or in response to novelty, may not necessarily be indicative of a heightened anxiety state in BTBR mice. Five weeks is just prior to puberty onset at around postnatal day 40 for most male mice, including strains such as C57BL/6 and 129S (Wisniewski et al., 2005; Qiu et al., 2013). Male puberty has not been characterized in BTBR mice, so it could occur earlier, as in strains such as CD1 or C3H/HeJ (Divall et al., 2010; Zhou et al., 2012), or later. Yet it is unlikely that any differences in puberty onset alone could have so distinguished the BTBR behavioral and neuroendocrine phenotype. Other endocrine abnormalities are more likely responsible, for example in BTBR mice serum levels of insulin, testosterone, and progesterone are also relatively high (Flowers et al.,

2007; Frye and Llaneza, 2010). Taken together with elevated baseline CORT levels and exaggerated stress response, there is evidence of pituitary over-activity, possibly due to feedback disruption in BTBR mice. These conditions can adversely impact cognition and behavior (Stranahan et al., 2008).

4.2. Age-dependent patterns of 5-HT_{1A} receptor regulation in BTBR mice

5-HT_{1A} receptors in the ventral and dorsal hippocampus shape social behavior, since 5-HT_{1A} agonists and antagonists affecting anxiety state were found to bi-directionally alter social interactions in rodent open-field tests (File et al., 1996; File and Seth, 2003). Indeed in a prior study we found that a low dose of buspirone (2 mg/kg) improved sociability in adult male BTBR mice (Gould et al., 2011), while a higher dose (10 mg/kg) worsened it (unpublished data). We also saw that 8-OH-DPAT stimulated GTPγS binding in the CA1 of hippocampus was higher in BTBR than in C57BL/10 adult male mice. Hence we sought to determine if our finding of enhanced hippocampal agonist-stimulated 5-HT_{1A} G-protein coupling would generalize to other socially impaired strains such as 129S, and if it was also evident in adolescent BTBR mice.

We found an age-dependent pattern of up-regulation at hippocampal 5-HT_{1A} receptors in BTBR relative to C57 mice. Specifically, higher [^{3}H] 8-OH-DPAT binding density was found in adolescents, while greater 8-OH-DPAT stimulated G-protein coupling was evident in adult BTBR mice as compared to age-matched C57 males. Yet 5-HT_{1A} receptor density did not differ between BTBR and C57 adults. The 129S mice also had higher [^{3}H] 8-OH-DPAT binding density, but only in the CA1 region of the hippocampus, and their 8-OH-DPAT stimulated GTPγS binding was similar to C57 mice.

4.3. Mounting evidence for possible GR dysfunction in BTBR mice

One way by which glucocorticoids modify behavior and HPA feedback response is via changes they generate at the neurotransmitter receptor level (e.g. Schutsky et al., 2011). However, the pattern of hippocampal 5-HT_{1A} expression and function in BTBR mice, taken together with other findings in the literature, indicates that their GR functional integrity may be compromised. For example, adrenalectomized rodents have increased hippocampal 5-HT_{1A} expression without changes in agonist-stimulated [^{35}S] GTPγS binding (Pompili et al., 2010), paralleling our findings in adolescent BTBR mice. Also, hippocampal GR are over-expressed in adult BTBR mice, yet baseline CORT levels are elevated (Silverman et al., 2010; Benno et al., 2009). Further, BTBR and 129S have relatively high thermal pain thresholds compared to C57BL/6 mice (Silverman et al., 2010; Hossain et al., 2004). Yet chronically elevated corticosteroids, as found in BTBR, typically increase pain intensity via GR-induced alterations in cellular signaling (Khasar et al., 2008; McEwen and Kalia, 2010; Tramullas et al., 2012). Finally, in GR+/− mice CORT administration enhanced 8-OH-DPAT-stimulated [^{3}H] GTPγS binding in the hippocampal CA1 (Hensler et al., 2010) to a similar extent to what we found in BTBR adults. Thus CORT levels may be higher due to compromised GR function in BTBR mice consequentially leading to increased 5-HT_{1A} function at the G-protein level.

4.4. Up-regulation of CB_1 function in the adult male BTBR dentate gyrus

Social behavior in inbred mice is also sensitive to synthetic cannabinoid agonists and manipulation of endogenous cannabinoid levels in frontal cortex and other brain regions by acetaminophen (Umathe et al., 2009; Gould et al., 2012). Given this, we compared CB1 receptor density and G-protein coupling capacity among strains in adolescent and adult male mice. We saw that CP55,940 stimulated G-protein coupling was enhanced in the dentate gyrus of BTBR adults relative to 129S adults and adolescent mice in all strains. We investigated this finding further, since a survey of neuropathological markers in BTBR mice revealed that neurogenesis in their dentate gyrus and glial fiber growth was altered (Stephenson et al., 2011). We saw no differences in neuronal density as quantified by NeuN labeling, DAPI staining, or CB1 receptor expression as quantified by autoradiography with [^{3}H] CP55,940 in the adult BTBR dentate gyrus. However, enhancement of CP55,940-stimulated G-protein coupling may yet be due to differences in neural vs. glial cell composition in this brain region (López-Gallardo et al., 2012). More numerous glial cells in BTBR adults, and subsequent increases in the actions of Gαi/o coupled CB_2 receptors expressed on them (Brusco et al., 2008) could account for enhanced G-protein coupling capacity in the dentate gyrus.

4.5. Relative down-regulation of CB_1 function in adolescent 129S hippocampi

Agonist-stimulated [^{35}S] GTPγS binding generally increased with age at hippocampal CB_1 receptors in all strains, while CB1 receptor density did not. 129S adolescents and BTBR mice had higher [^{3}H] CP55,940 binding density than C57 mice at both ages in the dentate gyrus and CA3 regions. Despite this, 129S adolescent mice had relatively lower CP55,940 stimulated GTPγS binding in all hippocampal areas than C57 or BTBR adolescents. This disconnect between CB1 density and function was not present in adult 129S mice, given our CP55,940 stimulated GTPγS binding data from all three hippocampal subregions, and earlier findings in the cingulate cortex (Gould et al., 2012).

Instead, it appears to be a distinct developmental strain difference in the dentate gyrus that may relate to stress reactivity impairments and fear-related memory impairments that have already been characterized in adult 129S1/SvImJ mice (Camp et al., 2012). Endocannabinoids, via CB_1 receptors can prevent the retention of inappropriate generalized fear responses in mice by interfering with hippocampal long-term potentiation and plasticity (Reich et al., 2008; Jacob et al., 2012), and 129S mice exhibit fear overgeneralization and motor activity impairments that have been attributed to elevated anxiety (Kalueff and Tuohimaa, 2004; Camp et al., 2012). It is also possible that the increased 5-HT_{1A} expression we found in the 129S hippocampus might occur to compensate for functional CB_1 impairments to some extent.

4.6. Age-dependent interactions among CB1 and 5-HT_{1A} in BTBR HPA axis regulation

Adolescent rodents might be less sensitive to cannabinoid-induced modulation of 5-HT_{1A} expression and function then adults (Zavitsanou et al., 2010). Hence while in the aggregate the functional G-protein binding response of both CB_1 and 5-HT_{1A} receptors is lower in BTBR adolescents than in adults, inhibitory feedback to the HPA axis may be muted. This could be why adolescent, but not adult CORT responses to novelty were exaggerated in BTBR mice. It is plausible that up-regulation of inhibitory coupling capacity at both 5-HT_{1A} and CB_1 receptors in these hippocampal regions is required to keep BTBR HPA axis activity in check and dampen the exaggerated adolescent novelty-induced CORT peak in BTBR adults. In turn, up-regulation of hippocampal 5-HT_{1A} and CB_1 receptor function or density in BTBR and 129S mice may similarly contribute to the lack of preference for social interaction in these strains.

4.7. Implications for strain selection in studies of social behavior

Poor emotional resilience in response to stress is associated with impulsive or aggressive behaviors, and psychiatric disorders such as anxiety and depression (Cuomo et al., 2008; Stevens et al., 2009). Impaired HPA axis feedback in susceptible individuals may contribute to this state (Sher, 2006; Spijker and van Rossum, 2012). Glucocorticoid tone may be particularly relevant to how the mature BTBR behavioral phenotype develops, since it shapes dendritic spine formation and pruning in the hippocampus (Liston and Gan, 2011; Gourley et al., 2013). This discovery is also potentially relevant for social interactions in teens with autism, since in controlled studies novel social stimuli raised cortisol levels higher in autistic than normotypic adolescents (Corbett et al., 2010; Schupp et al., 2013). In this sense, the BTBR mouse could provide critical mechanistic insight into how glucocorticoids shape adolescent social behavior. Given the variability in CORT response, 5-HT_{1A} and CB_1 receptor expression among BTBR, 129S and C57 mice, their use as a combined model system could help to clarify their effects on endpoints such as social behavior.

Role of the funding sources

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Conflict of interest statement

The authors have no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2013.09.003.

References

Atsak, P., Hauer, D., Campolongo, P., Schelling, G., McGaugh, J.L., Roozendaal, B., 2012. Glucocorticoids interact with the hippocampal endocannabinoid system in impairing retrieval of contextual fear memory. Proc. Natl. Acad. Sci. 109, 3504—3509.

Benno, R., Smirnova, Y., Vera, S., Liggett, A., Schanz, N., 2009. Exaggerated responses to stress in the BTBR T+tf/J mouse: an unusual behavioral phenotype. Behav. Brain. Res. 197, 462—465.

Brenner, A.B., Zimmerman, M.A., Bauermeister, J.A., Caldwell, C.H., 2013. The physiological expression of living in disadvantaged neighborhoods for youth. J. Youth Adolesc. 42, 792—806.

Brusco, A., Tagliaferro, P.A., Saez, T., Onaivi, E.S., 2008. Ultrastructural localization of neuronal brain CB_2 cannabinoid receptors. Ann. N.Y. Acad. Sci. 1139, 450—457.

Camp, M.C., Macpherson, K.P., Lederle, L., Graybeal, C., Gaburro, S., Debrouse, L.M., Ihne, J.L., Bravo, J.A., O'Connor, R.M., Ciocchi, S., Wellman, C.L., Lüthi, A., Cryan, J.F., Singewald, N., Holmes, A., 2012. Genetic strain differences in learned fear inhibition associated with variation in neuroendocrine, autonomic, and amygdala dendritic phenotypes. Neuropsychopharmacology 37, 1534—1547.

Corbett, B.A., Schupp, C.W., Simon, D., Ryan, N., Mendoza, S., 2010. Elevated cortisol during play is associated with age and social engagement in children with autism. Mol. Autism 1, 13.

Cota, D., 2008. The role of the endocannabinoid system in the regulation of hypothalamic-pituitary-adrenal axis activity. J. Neuroendocrinol. Suppl. 1, 35—38.

Cuomo, C., Sarchiapone, M., Giannantonio, M.D., Mancini, M., Roy, A., 2008. Aggression, impulsivity, personality traits, and childhood trauma of prisoners with substance abuse and addiction. Am. J. Drug Alcohol Abuse 34, 339—345.

Divall, S.A., Williams, T.R., Carver, S.E., Koch, L., Brüning, J.C., Kahn, C.R., Wondisford, F., Radovick, S., Wolfe, A., 2010. Divergent roles of growth factors in the GnRH regulation of puberty in mice. J. Clin. Invest. 120, 2900—2909.

File, S.E., Gonzalez, L.E., Andrews, N., 1996. Comparative study of pre- and postsynaptic 5-HT_{1A} receptor modulation of anxiety in two ethological animal tests. J. Neurosci. 16, 4810—4815.

File, S.E., Seth, P., 2003. A review of 25 years of the social interaction test. Eur. J. Pharmacol. 463, 35—53.

Flowers, J.B., Oler, A.T., Nadler, S.T., Choi, Y., Schueler, K.L., Yandell, B.S., Kendziorski, C.M., Attie, A.D., 2007. Abdominal obesity in BTBR male mice is associated with peripheral but not hepatic insulin resistance. Am. J. Physiol. Endocrinol. Metab. 292, E936—E945.

Frye, C.A., Llaneza, D.C., 2010. Corticosteroid and neurosteroid dysregulation in an animal model of autism BTBR mice. Physiol. Behav. 100, 264—267.

Garlow, S.J., Rosenberg, J., Moore, J.D., Haas, A.P., Koestner, B., Hendin, H., Nemeroff, C.B., 2008. Depression, desperation, and suicidal ideation in college students: results from the American Foundation for Suicide Prevention College Screening Project at Emory University. Depress. Anxiety 25, 482–488.

Gould, G.G., Hensler, J.G., Burke, T.F., Benno, R.H., Onaivi, E.S., Daws, L.C., 2011. Density and function of central serotonin (5-HT) transporters, 5-HT_{1A} and 5-HT_{2A} receptors, and effects of their targeting on BTBR T+tf/J mouse social behavior. J. Neurochem. 116, 291–303.

Gould, G.G., Seillier, A., Weiss, G., Giuffrida, A., Burke, T.F., Hensler, J.G., Rock, C., Tristan, A., McMahon, L.R., Salazar, A., O'Connor, J.C., Satsangi, N., Satsangi, R.K., Gu, T.T., Treat, K., Smolik, C., Schultz, S.T., 2012. Acetaminophen differentially enhances social behavior and cortical cannabinoid levels in inbred mice. Prog. Neuropsychopharmacol. Biol. Psychiatry 38, 260–269.

Gourley, S.L., Swanson, A.M., Koleske, A.J., 2013. Corticosteroid-induced neural remodeling predicts behavioral vulnerability and resilience. J. Neurosci. 33, 3107–3112.

Hackman, D.A., Betancourt, L.M., Brodsky, N.L., Hurt, H., Farah, M.J., 2012. Neighborhood disadvantage and adolescent stress reactivity. Front. Hum. Neurosci. 6, 277.

Hensler, J.G., Vogt, M.A., Gass, P., 2010. Regulation of cortical and hippocampal 5-HT (1A) receptor function by corticosterone in GR+/− mice. Psychoneuroendocrinology 35, 469–474.

Hossain, S.M., Wong, B.K., Simpson, E.M., 2004. The dark phase improves genetic discrimination for some high throughput mouse behavioral phenotyping. Genes Brain Behav. 3, 167–177.

Jacob, W., Marsch, R., Marsicano, G., Lutz, B., Wotjak, C.T., 2012. Cannabinoid CB1 receptor deficiency increases contextual fear memory under highly aversive conditions and long-term potentiation in vivo. Neurobiol. Learn. Mem. 98, 47–55.

Kalueff, A.V., Tuohimaa, P., 2004. Contrasting grooming phenotypes in C57Bl/6 and 129S1/SvImJ mice. Brain Res. 1028, 75–82.

Khasar, S.G., Burkham, J., Dina, O.A., Brown, A.S., Bogen, O., Alessandri-Haber, N., Green, P.G., Reichling, D.B., Levine, J.D., 2008. Stress induces a switch of intracellular signaling in sensory neurons in a model of generalized pain. J. Neurosci. 28, 5721–5730.

Lanfumey, L., Mongeau, R., Cohen-Salmon, C., Hamon, M., 2008. Corticosteroid–serotonin interactions in the neurobiological mechanisms of stress-related disorders. Neurosci. Biobehav. Rev. 32, 1174–1184.

Liston, C., Gan, W.B., 2011. Glucocorticoids are critical regulators of dendritic spine development and plasticity in vivo. Proc. Natl. Acad. Sci. U.S.A. 108, 16074–16079.

Lopez-Duran, N.L., Kovacs, M., George, C.J., 2009. Hypothalamic-pituitary-adrenal axis dysregulation in depressed children and adolescents: a meta-analysis. Psychoneuroendocrinology 34, 1272–1283.

López-Gallardo, M., López-Rodríguez, A.B., Llorente-Berzal, Á., Rotllant, D., Mackie, K., Armario, A., Nadal, R., Viveros, M.P., 2012. Maternal deprivation and adolescent cannabinoid exposure impact hippocampal astrocytes, CB1 receptors and brain-derived neurotrophic factor in a sexually dimorphic fashion. Neuroscience 204, 90–103.

McCormick, C.M., Mathews, I.Z., 2007. HPA function in adolescence: role of sex hormones in its regulation and the enduring consequences of exposure to stressors. Pharmacol. Biochem. Behav. 86, 220–233.

McLaughlin, R.J., Hill, M.N., Gorzalka, B.B., 2009. Monoaminergic neurotransmission contributes to cannabinoid-induced activation of the hypothalamic-pituitary-adrenal axis. Eur. J. Pharmacol. 624, 71–76.

McEwen, B.S., Kalia, M., 2010. The role of corticosteroids and stress in chronic pain conditions. Metabolism 59 (Suppl. 1) S9–S15.

Moy, S.S., Nadler, J.J., Young, N.B., Perez, A., Holloway, L.P., Barbaro, R.P., Barbaro, J.R., Wilson, L.M., Threadgill, D.W., Lauder, J.M., Magnuson, T.R., Crawley, J.N., 2007. Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains. Behav. Brain Res. 176, 4–20.

Pompili, M., Serafini, G., Innamorati, M., Möller-Leimkühler, A.M., Giupponi, G., Girardi, P., Tatarelli, R., Lester, D., 2010. The hypothalamic-pituitary-adrenal axis and serotonin abnormalities: a selective overview for the implications of suicide prevention. Eur. Arch. Psychiatry Clin. Neurosci. 260, 583–600.

Pryce, C., 2008. Postnatal ontogeny of expression of the corticosterone receptor genes in mammalian brains: inter-species and intra-species differences. Brain Res. Rev. 57, 596–605.

Qiu, X., Dowling, A.R., Marino, J.S., Faulkner, L.D., Bryant, B., Brüning, J.C., Elias, C.F., Hill, J.W., 2013. Delayed puberty but normal fertility in mice with selective deletion of insulin receptors from Kiss1 cells. Endocrinology 154, 1337–1348.

Reich, C.G., Mohammadi, M.H., Alger, B.E., 2008. Endocannabinoid modulation of fear responses: learning and state-dependent performance effects. J. Psychopharmacol. 22, 769–777.

Romeo, R.D., Kaplowitz, E.T., Ho, A., Franco, D., 2013. The influence of puberty on stress reactivity and forebrain glucocorticoid receptor levels in inbred and outbred strains of male and female mice. Psychoneuroendocrinology 38, 592–596.

Schupp, C.W., Simon, D., Corbett, B.A., 2013. Cortisol responsivity differences in children with autism spectrum disorders during free and cooperative play. J. Autism Dev. Disord. 43, 2405–2417.

Schutsky, K., Ouyang, M., Castelino, C.B., Zhang, L., Thomas, S.A., 2011. Stress and glucocorticoids impair memory retrieval via β2-adrenergic, Gi/o-coupled suppression of cAMP signaling. J. Neurosci. 31, 14172–14181.

Sher, L., 2006. Combined dexamethasone suppression-corticotropin-releasing hormone stimulation test in studies of depression, alcoholism, and suicidal behavior. Scientific World J. 6, 1398–1404.

Silverman, J.L., Yang, M., Turner, S.M., Katz, A.M., Bell, D.B., Koenig, J.I., Crawley, J.N., 2010. Low stress reactivity and neuroendocrine factors in the BTBR T+tf/J mouse model of autism. Neuroscience 171, 1197–1208.

Spijker, A.T., van Rossum, E.F., 2012. Glucocorticoid sensitivity in mood disorders. Neuroendocrinology 95, 179–186.

Spratt, E.G., Nicholas, J.S., Brady, K.T., Carpenter, L.A., Hatcher, C.R., Meekins, K.A., Furlanetto, R.W., Charles, J.M., 2012. Enhanced cortisol response to stress in children in autism. J. Autism Dev. Disord. 42, 75–81.

Stevens, H.E., Leckman, J.F., Coplan, J.D., Suomi, S.J., 2009. Risk and resilience: early manipulation of macaque social experience and persistent behavioral and neurophysiological outcomes. J. Am. Acad. Child Adolesc. Psychiatry 48, 114–127.

Stephenson, D.T., O'Neill, S.M., Narayan, S., Tiwari, A., Arnold, E., Samaroo, H.D., Du, F., Ring, R.H., Campbell, B., Pletcher, M., Vaidya, V.A., Morton, D., 2011. Histopathologic characterization of the BTBR mouse model of autistic-like behavior reveals selective changes in neurodevelopmental proteins and adult hippocampal neurogenesis. Mol. Autism 2, 7.

Stranahan, A.M., Lee, K., Pistell, P.J., Nelson, C.M., Readal, N., Miller, M.G., Spangler, E.L., Ingram, D.K., Mattson, M.P., 2008. Accelerated cognitive aging in diabetic rats is prevented by lowering corticosterone levels. Neurobiol. Learn. Mem. 90, 479–483.

Sunnqvist, C., Westrin, A., Träskman-Bendz, L., 2008. Suicide attempters: biological stressmarkers and adverse life events. Eur. Arch. Psychiatry Clin. Neurosci. 258, 456–462.

Tramullas, M., Dinan, T.G., Cryan, J.F., 2012. Chronic psychosocial stress induces visceral hyperalgesia in mice. Stress 15, 281–292.

Umathe, S.N., Manna, S.S., Utturwar, K.S., Jain, N.S., 2009. Endocannabinoids mediate anxiolytic-like effect of acetaminophen via CB1 receptors. Prog. Neuropsychopharmacol. Biol. Psychiatry 33, 1191–1199.

Wisniewski, A.B., Cernetich, A., Gearhart, J.P., Klein, S.L., 2005. Perinatal exposure to genistein alters reproductive development and aggressive behavior in male mice. Physiol. Behav. 84, 327—334.

Zavitsanou, K., Wang, H., Dalton, V.S., Nguyen, V., 2010. Cannabinoid administration increases $5HT_{1A}$ receptor binding and mRNA expression in the hippocampus of adult but not adolescent rats. Neuroscience 169, 315—324.

Zhou, Y., Guan, Q., Li, K., Tao, L., Hu, J., Xiao, J., 2012. Dissection of the maternal effects on puberty onset by embryo transplantation in mouse. J. Endocrinol. Invest. 35, 676—680.

Systems/Circuits

Decynium-22 Enhances SSRI-Induced Antidepressant-Like Effects in Mice: Uncovering Novel Targets to Treat Depression

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Mood disorders cause much suffering and lost productivity worldwide, compounded by the fact that many patients are not effectively treated by currently available medications. The most commonly prescribed antidepressant drugs are the selective serotonin (5-HT) reuptake inhibitors (SSRIs), which act by blocking the high-affinity 5-HT transporter (SERT). The increase in extracellular 5-HT produced by SSRIs is thought to be critical to initiate downstream events needed for therapeutic effects. A potential explanation for their limited therapeutic efficacy is the recently characterized presence of low-affinity, high-capacity transporters for 5-HT in brain [i.e., organic cation transporters (OCTs) and plasma membrane monoamine transporter], which may limit the ability of SSRIs to increase extracellular 5-HT. Decynium-22 (D-22) is a blocker of these transporters, and using this compound we uncovered a significant role for OCTs in 5-HT uptake in mice genetically modified to have reduced or no SERT expression (Baganz et al., 2008). This raised the possibility that pharmacological inactivation of D-22-sensitive transporters might enhance the neurochemical and behavioral effects of SSRIs. Here we show that in wild-type mice D-22 enhances the effects of the SSRI fluvoxamine to inhibit 5-HT clearance and to produce antidepressant-like activity. This antidepressant-like activity of D-22 was attenuated in OCT3 KO mice, whereas the effect of D-22 to inhibit 5-HT clearance in the CA3 region of hippocampus persisted. Our findings point to OCT3, as well as other D-22-sensitive transporters, as novel targets for new antidepressant drugs with improved therapeutic potential.

Introduction

Dysfunction of the serotonin (5-HT) system is thought to underlie many affective disorders, primary among them, depression (Charney et al., 1981). The most commonly prescribed antidepressant medications are selective serotonin reuptake inhibitors (SSRIs). SSRIs act to block activity of the high-affinity serotonin transporter (SERT) and prevent the uptake of 5-HT from extracellular fluid into nerve terminals (Blakely et al., 1998). The ensuing increase in extracellular 5-HT is considered to be an important element in triggering the downstream events needed to produce therapeutic effects. However, a major problem in the treatment of depression is that many patients experience modest or no therapeutic benefit (Kirsch et al., 2008), indicating the need for alternative approaches to treat individuals who respond poorly to SSRIs.

Organic cation transporters (i.e., OCT1, OCT2, and OCT3) and the plasma membrane monoamine transporter (PMAT) are low-affinity transporters for 5-HT, but unlike SERT, have a high capacity to transport 5-HT (Gründemann et al., 1998; Engel et al., 2004; Amphoux et al., 2006; Koepsell et al., 2007; Baganz et al., 2008; Duan and Wang, 2010). Decynium-22 (D-22) (originally described by Schömig et al., 1993), is a blocker of OCTs and PMAT, which inhibits 5-HT uptake into cell lines expressing OCT1, OCT2, OCT3, or PMAT as well as 5-HT uptake into brain synaptosomes (Engel et al., 2004; Engel and Wang, 2005; Gasser et al., 2006; Schömig et al., 2006; Duan and Wang, 2010; Hagan et al., 2011). These reports raise the possibility that low-affinity, high-capacity D-22-sensitive transporters for 5-HT may account, at least in part, for the poor clinical efficacy of SSRIs by preventing extracellular 5-HT levels reaching those required to trigger the cascade of downstream events needed for therapeutic benefit (Daws et al., 2013).

Support for this idea comes from studies in mice genetically modified to lack SERT or to express half as many SERTs as wild-type mice. In these SERT mutant mice, administration of D-22 inhibits 5-HT clearance from extracellular fluid in hippocampus and produces antidepressant-like effects (Baganz et al., 2008). In

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contrast, D-22 does not produce these effects in wild-type mice, perhaps not surprisingly, since these mice have a full complement of functioning SERT controlling 5-HT uptake. Thus it appears that D-22-sensitive uptake mechanisms become more important in regulating 5-HT uptake and behavior when SERT function is genetically inactivated or impaired (Baganz et al., 2008). This raises the possibility that D-22-sensitive transporters for 5-HT may also play a more prominent role in 5-HT clearance when the high-affinity SERT is pharmacologically inactivated. If so, by limiting the increase of 5-HT in extracellular fluid that follows treatment with an SSRI, D-22-sensitive transporters could provide a mechanistic basis for poor therapeutic outcome in many patients. Thus, blockade of D-22-sensitive transporters might be a novel way to increase the therapeutic efficacy of currently available antidepressant drugs. Here we report that D-22 augments the ability of the SSRI, fluvoxamine, to inhibit 5-HT uptake and to produce antidepressant-like effects, providing support for D-22-sensitive transporters as novel targets for new antidepressant drugs.

Materials and Methods

Animals. Adult (>60-d-old; 25–30 g) male C57BL/6 mice, or OCT3 knock-out (KO) mice (bred on a C57BL/6 background, originally developed by Zwart et al. (2001) and generously provided to us by Drs. Kim Tieu, Bruno Giros, and Sophie Gautron) were used for all experiments. All animal procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All measures were taken to limit the number of animals used and to minimize animal discomfort.

High-speed chronoamperometry. In vivo chronoamperometry was performed according to methods described in detail previously (Daws and Toney, 2007; Baganz et al., 2008). We assemble our own carbon fiber electrodes and a detailed description can be found in Williams et al. (2007). Recording electrode/micropipette assemblies were constructed using a single carbon fiber (30 μm diameter; Specialty Materials), which was sealed inside fused silica tubing (Schott, North America). We based our procedure for electrode construction on modifications of published methods (Gerhardt, 1995; Perez and Andrews, 2005). Carbon fiber electrodes were coated with Nafion (5% solution; Aldrich Chemical), to prevent interference from anionic substances in extracellular fluid as previously described (Daws and Toney, 2007). Electrodes were tested for sensitivity to the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA; 250 μM) and calibrated with accumulating concentrations of 5-HT (0–3 μM). Only electrodes displaying a selectivity ratio for 5-HT over 5-HIAA greater than 500:1 and a linear response ($r^2 \geq 0.9$) to 5-HT were used.

The electrochemical recording assembly consisted of a Nafion-coated, single carbon fiber electrode attached to a four-barreled micropipette such that their tips were separated by ~200 μm. Barrels were filled with combinations of either 5-HT (200 μM), D-22 (10 μM), fluvoxamine (400 μM), a combination of D-22 and fluvoxamine, or phosphate-buffered saline (PBS). We elected to use fluvoxamine primarily to allow direct comparison with over a decade of chronoamperometry data reported in the literature, including those from our own lab, as well as others who have used fluvoxamine routinely (Daws et al., 1998; Benmansour et al., 1999, 2002, 2012; Montañez et al., 2003; Daws et al., 2005; Baganz et al., 2008). All compounds were prepared in 0.1 M PBS with 100 μM ascorbic acid added as an antioxidant and the pH adjusted to 7.4. The electrode-micropipette recording assembly was lowered into the CA3 region of the dorsal hippocampus (anteroposterior, −1.94 from bregma; mediolateral, +2.0 from midline; dorsoventral, −2.0 from dura) (Franklin and Paxinos, 1997) of anesthetized mice. For all experiments, mice were anesthetized by intraperitoneal injection (2 ml/kg body weight) of a mixture of chloralose (35 mg/kg) and urethane (350 mg/kg). A tube was inserted into the trachea to facilitate breathing and mice were then placed into a stereotaxic frame. Body temperature was maintained at 36−37°C by a water circulated heating pad.

High-speed chronoamperometric recordings were made using the FAST-12 and FAST-16 systems (Quanteon). Oxidation potentials consisted of 100 ms pulses of +0.55 V. Each pulse was separated by a 900 ms interval during which the resting potential was maintained at 0.0 V. Voltage at the active electrode was applied with respect to an Ag/AgCl reference electrode positioned in the extracellular fluid of the ipsilateral superficial cortex. The oxidation and reduction currents were digitally integrated during the last 80 ms of each 100 ms voltage pulse.

At the conclusion of the experiment, an electrolytic lesion was made to mark the placement of the electrode tip. The brain was removed, rapidly frozen on dry ice, and stored at −80°C until use. At this time brains were thawed to −15°C and sliced into 20-μm-thick sections for histological verification of electrode localization. Only data from mice in which the electrode was confirmed to be in the CA3 region of the hippocampus were included in the analyses.

Effects of local and systemic drug administration on 5-HT clearance. Exogenous 5-HT was applied into the CA3 region of the hippocampus by pressure ejection (5–25 psi for 0.25–3.0 s). Advantages of this approach are that clearance can be measured without an associated "release" component and that measurements can be made *in vivo* with excellent temporal (milliseconds) resolution. Serotonin was pressure ejected into hippocampus to achieve concentrations at the recording electrode of ~0.5 μM. Once reproducible 5-HT electrochemical signals were obtained, drug or vehicle was applied either locally into the CA3 region of hippocampus, or intraperitoneally, before the next application of 5-HT. For locally administered drugs, 5-HT was pressure ejected 2 min after drug or vehicle to allow sufficient time for drugs to diffuse to the recording site. Serotonin was applied again at 10 min intervals after drug or vehicle until the signal returned to predrug status. The 10 min time interval ensured that each signal produced by local application of 5-HT had completely cleared before the next ejection of 5-HT, drug or vehicle. For systemic administration, exogenous 5-HT was applied 30 min following intraperitoneal administration of drugs or vehicle so as to correspond to the timing used in the tail suspension test (TST). Two signal parameters were analyzed: the peak signal amplitude and the T_{80} time course parameter. T_{80} is defined as the time for the signal to decline by 80% of the peak signal amplitude.

TST. The TST was conducted based on the original description by Steru et al. (1985). In dose–response studies, mice received an intraperitoneal injection of D-22 (0.01–0.32 mg/kg), fluvoxamine (0.32–32 mg/kg), or saline. In drug interaction studies, mice received an intraperitoneal injection of either D-22 (0.1 or 0.32 mg/kg) or saline 60 min before testing, and received a second intraperitoneal injection of either fluvoxamine (10 mg/kg) or saline 30 min before testing; immediately after each injection, they were placed in an observation cage. Thirty minutes after the second injection, the distal end of the tail was fastened to a flat aluminum (2 × 0.3 × 10 cm) bar using adhesive tape at a 90 degree angle to the longitudinal axis of the mouse tail and the aluminum bar, with a distance of 3–4 cm between the base of the tail and the edge of the bar. A hole opposite the taped end of the bar was used to secure the bar to a hook in the ceiling of a visually isolated white test box (40 × 40 × 40 cm). Each mouse was suspended by its tail for 6 min, allowing the ventral surface and front and hindlimbs to be video recorded using a digital camera facing the test box. Total time immobile was measured (in seconds) during the entire 6 min test period. Immobility was defined as the absence of initiated movements, and included passive swaying of the body. A mouse was excluded from the experiments if it climbed and held to its tail or the aluminum bar for a period of 3 s or longer.

Serotonin syndrome. Mice were given saline or a combination of D-22 and fluvoxamine, using the same doses and injection-test intervals as used in the drug interaction study in the TST. In addition, separate groups of mice received 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT, 10 mg/kg), 5-hydroxy-L-tryptophan (5-HTP, 100 mg/kg), or their vehicles (saline and 5% Tween 80, respectively). Each of the five groups received two intraperitoneal injections, 30 min apart, as follows: (1) D-22 followed by fluvoxamine, (2) saline followed by 8-OH-DPAT, (3) saline followed by 5-HTP, (4) saline followed by saline and (5) saline followed by 5% Tween 80. Immediately after the first injection, animals

were placed in a transparent observation cage with a mirror positioned beneath it to allow simultaneous viewing of both the side and ventral surface of the mouse. Mice were observed for possible occurrence of elements of the 5-HT syndrome during five, 6 min periods, starting at 15, 30, 60, 120, and 240 min after the second injection. Each animal was recorded on video until immediately after the observation period that started at 120 min. The animal was then returned to the home cage where it was observed at 240 min. During each 6 min period mice were observed for the occurrence of the following signs: hindlimb abduction, low body posture, Straub tail, backward movement, tremor, head weaving, and forepaw treading, according to methods adapted from Fox et al. (2008). The presence (1) or absence (0) of each sign was assessed during each min of the 6 min observation period, allowing an animal to receive, for each of the signs, a score of 0–6 at each observation period and an overall score of 30 for all five observation periods.

HPLC analysis of D-22 in brain. HPLC was used to assay for D-22 in brain. D-22 (10 mg/kg) was injected intraperitoneally and mice were killed 30 min later. Brain tissue for samples, calibrators, and controls were weighed into polypropylene tubes, then 10 times the volume of a 70% acetonitrile solution was added. Samples were homogenized by sonication and then 500 μl of each was placed in clean polypropylene tubes. Twenty microliters of diazepam (10 μg/ml) for internal standard was added to each sample. Next, samples were shaken for 10 min, centrifuged at 3200 *g* for 20 min at 18°C in a Beckman Allegra X-15R centrifuge. The supernatant was decanted into clean tubes, and dried under a stream of nitrogen. Samples were resuspended with 100 μl of mobile phase buffer, and microfilterfuged at 3200 *g* for 10 min. Fifty microliters of sample was injected into the HPLC system.

The HPLC system included a Waters model 510 pump, a Waters model 717 sample injector, a Waters model 2487 UV detector for HPLC equipped with a deuterium lamp (320/520 nm), and an Alltima 150 mm × 4.6 mm C18 column (5 U). Samples were analyzed at a fixed wavelength of 520 nm, a time constant of 1 s, and a range of 0.1 AUFS on the UV detector. The mobile phase contained 65% methanol, and 35% of a solution of 12 mM KH_2PO_4, pH 6.7. The flow rate of the mobile phase was 1.5 ml/min. D-22 eluted at 8.7 min and diazepam at 10.1 min. The peak area ratio of D-22 and the internal standard diazepam were determined using the Waters Empower chromatography software. D-22 concentration was determined by comparing peak area ratio (D22/diazepam) against the linear regression of ratios of calibrator samples from a 6 point calibrator curve. The concentrations of D-22 in brain were expressed as pg D-22/mg brain wet weight.

High purity-grade acetonitrile and methanol were purchased from Burdick and Jackson. Water used in the assay was purified with a Milli-Q Water System (Millipore). Potassium phosphate was obtained from Fisher Scientific. Hydrochloric acid was obtained from JT Baker Chemical. The internal standard, diazepam, was obtained from Sigma.

Statistics. Statistical analyses were performed using Prism 5.04 (GraphPad). Data were analyzed using one-way ANOVA, followed by Newman–Keuls or Dunnett's multiple-comparison tests. All data were expressed as mean ± SEM, and $p < 0.05$ was considered statistically significant.

Drugs. Serotonin, 5-HIAA, fluvoxamine, urethane, α-chloralose, D-22, 8-OH-DPAT HBr, and 5-HTP were purchased from Sigma-Aldrich. All compounds were injected intraperitoneally in a volume of 5–20 ml/kg. Doses are expressed as mg salt weight per kg body weight.

Results

Intrahippocampally applied D-22 enhances the ability of the SSRI fluvoxamine to inhibit 5-HT clearance from extracellular fluid

In wild-type mice, we previously found that D-22 could inhibit 5-HT clearance from CA3 region of hippocampus, but only when extracellular 5-HT levels were in a range that recruited low-affinity, high-capacity transporters for 5-HT (Baganz et al., 2008, 2010). At lower, SERT-recruiting 5-HT concentrations, D-22 had no effect on 5-HT clearance, a result not especially surprising given the high affinity of SERT for 5-HT. However, in the presence of an SSRI, we reasoned that D-22-sensitive transporters may exert a more prominent role in 5-HT uptake and therefore, limit the ability of SSRIs to prolong the time 5-HT remains in extracellular fluid. This led us to hypothesize that D-22 should enhance the inhibiting effect of an SSRI on 5-HT clearance. To test this hypothesis we used high-speed chronoamperometry, according to well established protocols (Daws and Toney, 2007; Baganz et al., 2008, 2010), to measure clearance of locally applied 5-HT from the CA3 region of the hippocampus of anesthetized mice, before and after intrahippocampal administration of fluvoxamine, D-22, both drugs given together, or vehicle. The amount of fluvoxamine delivered (54 pmol) was based on previous dose–response studies showing maximal inhibition of 5-HT clearance at this dose (Baganz et al., 2010). The amount of D-22 delivered (1.4 pmol) is the maximum that can be delivered locally without interfering with the properties of the carbon fiber recording electrode. D-22 (1.4 pmol) has no effect on 5-HT clearance in the CA3 region of hippocampus in wild-type mice but inhibits 5-HT clearance in mice lacking SERT, suggesting that the 5-HT clearance inhibiting effect of D-22 is mediated at sites other than SERT, putatively OCT3 (Baganz et al., 2008). Serotonin was pressure ejected to achieve reproducible signals with amplitudes of ~0.5 μM, a concentration that we have previously found to recruit predominantly SERT-mediated 5-HT clearance (Daws et al., 2005; Baganz et al., 2008, 2010). Once reproducible signals were obtained, drug or vehicle was locally ejected into hippocampus and then 2 min later 5-HT was applied again. Representative traces are shown in Figure 1*A*. As expected, and consistent with other reports using similar methodology (Daws et al., 1998, 2000, 2005; Benmansour et al., 1999, 2002), fluvoxamine prolonged 5-HT clearance time (Fig. 1*A*,*B*), without producing any significant effect on signal amplitude (Fig. 1*A*,*C*). Likewise, replicating our previous study (Baganz et al., 2008), D-22 was without effect on 5-HT clearance time or signal amplitude (Fig. 1*A*–*C*). However, D-22 robustly increased the ability of fluvoxamine to inhibit 5-HT clearance. This effect was apparent not only in terms of prolonging 5-HT clearance time (Fig. 1*A*,*B*), but also in terms of increasing the amplitude of the 5-HT signal (Fig. 1*A*,*C*). Baseline (i.e., predrug) T_{80} values did not differ significantly ($p > 0.20$) among groups and were 101 ± 9, 116 ± 8, 107 ± 15, and 114 ± 6 s for vehicle, D-22, fluvoxamine, and D-22+fluvoxamine groups, respectively. One-way ANOVA revealed an effect of treatment on the percentage change from baseline 5-HT clearance time, driven by significant effects of fluvoxamine, and the combination of fluvoxamine and D-22 to slow 5-HT clearance ($F_{(3,35)} = 12.07$; $p < 0.0001$; Fig. 1*A*,*B*). Likewise, baseline (i.e., predrug) signal amplitude values did not differ significantly ($p > 0.50$) among groups and were 0.66 ± 0.04, 0.56 ± 0.04, 0.65 ± 0.04, and 0.58 ± 0.06 μM for vehicle, D-22, fluvoxamine, and D-22+fluvoxamine groups, respectively. One-way ANOVA revealed an effect of treatment on the percentage change from baseline in 5-HT signal amplitude, driven by the marked ability of fluvoxamine, in combination with D-22, to increase signal amplitude ($F_{(3,35)} = 3.85$; $p < 0.05$; Fig. 1*A*,*C*).

It is worth noting that we conducted these *in vivo* electrochemical studies in the hippocampus for three key reasons. First, it is a limbic structure considered to be important in the therapeutic response to antidepressant drugs (Campbell and Macqueen, 2004). Second, it is a region where mechanisms contributing to 5-HT clearance *in vivo* have been best characterized to date (Daws et al., 1998, 2000, 2005; Baganz et al., 2008). Third, and especially relevant to the present study are reports that D-22-sensitive OCTs and PMAT are located in the hippocampus. Indeed, the availability of selective antibodies has made it possible

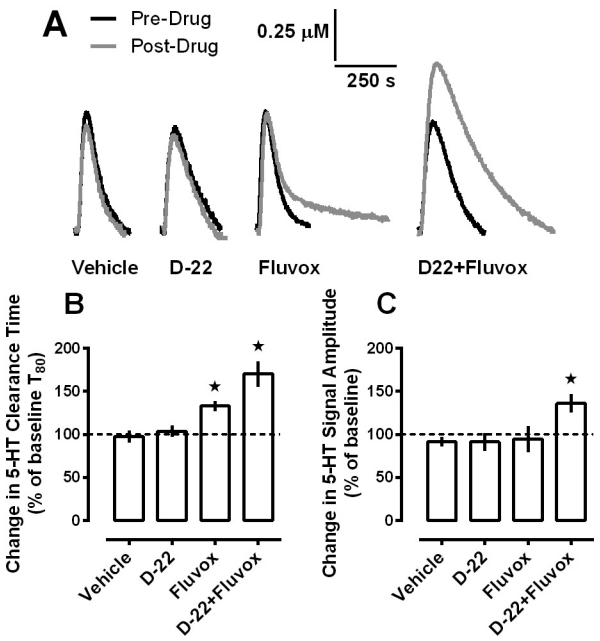

Figure 1. Local blockade of D-22-sensitive transporters enhances fluvoxamine-induced inhibition of 5-HT clearance in mouse hippocampus *in vivo*. ***A***, Representative oxidation currents (converted to micromolar values) produced by pressure ejection of 5-HT into the CA3 region of the hippocampus before (black line) and 2 min after (gray line) local application of saline, D-22 (1.4 pmol), fluvoxamine (54 pmol), or fluvoxamine and D-22 given together. Raw tracings are superimposed for ease of comparison. Note the marked increase in both clearance time and signal amplitude after local application of D-22 and fluvoxamine when given together. ***B***, The effect of hippocampally applied fluvoxamine to slow 5-HT clearance is enhanced by concurrent administration of D-22; one-way ANOVA with Newman–Keuls *post hoc* comparison, $*p < 0.05$ versus vehicle. ***C***, Hippocampally applied fluvoxamine, in combination with D-22, increases 5-HT signal amplitude; one-way ANOVA with Newman–Keuls *post hoc* comparison, $*p < 0.05$ versus vehicle. Data are expressed as mean ± SEM percentage of baseline values with $n = 9–10$ for each drug treatment.

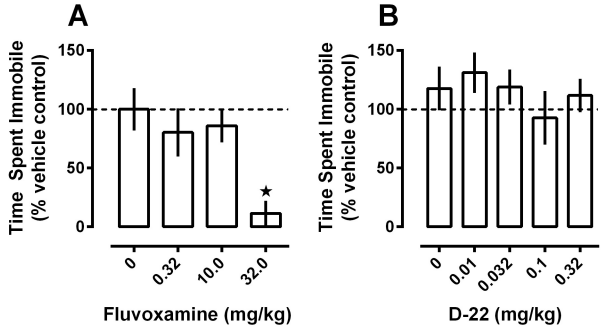

Figure 2. Fluvoxamine, but not D-22, dose dependently reduces immobility time in the TST. Time spent immobile was measured in seconds, and was expressed as a percentage of the corresponding vehicle control group. Time spent immobile for vehicle control groups did not differ significantly ($p > 0.50$) and were 116 ± 19 and 118 ± 19 s for fluvoxamine and D-22 control groups, respectively. ***A***, Fluvoxamine, administered 30 min before testing, dose dependently decreased time spent immobile, producing a near maximal effect at the highest dose tested. ***B***, D-22, given 60 min before testing, had no significant effect on time spent immobile at any of the doses tested. Data are expressed as mean ± SEM, $n = 7–9$ for each dose tested; one-way ANOVA with Dunnett's multiple-comparison *post hoc* test, $*p < 0.05$.

to map the distribution of OCT subtypes and PMAT in brain (Amphoux et al., 2006; Dahlin et al., 2007; Vialou et al., 2007; Baganz et al., 2008; Lamhonwah et al., 2008; Cui et al., 2009; Gasser et al., 2009; Bacq et al., 2012).

The finding that locally administered D-22 can markedly enhance fluvoxamine-induced inhibition of 5-HT clearance in hippocampus suggests that the combination of these drugs might produce similar effects on 5-HT clearance when administered systemically and, importantly, enhance the antidepressant-like effect of fluvoxamine. The next set of experiments was designed to test this hypothesis.

D-22 enhances fluvoxamine-induced antidepressant-like effects in the TST

The ability of intrahippocampally applied D-22 to enhance fluvoxamine-induced inhibition of 5-HT clearance supports the idea that this drug combination might also produce greater antidepressant-like effects than either drug given alone. To this end, we studied the effects of systemic administration of D-22 and fluvoxamine in the TST, which measures antidepressant-like activity in mice (Steru et al., 1985; Cryan et al., 2005). We then examined how time spent immobile in the TST related to drug-induced inhibition of 5-HT clearance in hippocampus.

Consistent with the literature (Fujishiro et al., 2002; Cryan et al., 2005), fluvoxamine, given 30 min before testing, dose dependently reduced time spent immobile in the TST (Fig. 2*A*; $F_{(3,28)} = 5.75$, $p < 0.01$). Previously, we found that 0.001 mg/kg D-22 decreased immobility in SERT-deficient mice, but not in wild-type mice, and decreased immobility more extensively at 60 min than at 30 min after intraperitoneal administration (Baganz et al., 2008). Here, we examined the effects of D-22 60 min after intraperitoneal administration in wild-type mice at doses ranging from 0.01 to 0.32 mg/kg. At these doses, D-22 did not significantly affect immobility (Fig. 2*B*; $p > 0.60$). This result was not especially surprising, given the presence of fully functioning, high-affinity SERT in these mice, which serve to maintain extracellular 5-HT at concentrations below those where low-affinity, high-capacity transporters for 5-HT can exert more influence. Doses >0.32 mg/kg, which in preliminary experiments appeared to decrease locomotion, were not tested.

To test our hypothesis that D-22 would enhance the antidepressant-like effect of fluvoxamine, we selected a dose of each drug that did not produce significant effects on time spent immobile in the TST (i.e., 0.1 mg/kg D-22, and 10 mg/kg fluvoxamine given 60 and 30 min before testing, respectively). Consistent with the results shown in Figure 2, treatment with either D-22 (0.1 mg/kg i.p.) or fluvoxamine (10 mg/kg, i.p.) alone produced no statistically significant decrease in time spent immobile relative to saline-treated control mice (Fig. 3*A*). However, cotreatment with D-22 and fluvoxamine markedly decreased time spent immobile in the TST (one-way ANOVA, $F_{(3,31)} = 6.92$, $p < 0.05$).

To examine whether the marked antidepressant-like effect of the combination of D-22 and fluvoxamine was related to an increased ability of this drug combination to inhibit 5-HT uptake, we used high-speed chronoamperometry to study 5-HT clearance in the CA3 region of hippocampus under the same doses and injection-test intervals as used in the TST. Consistent with behavioral data in Figure 3*A*, D-22 had no effect on clearance of intrahippocampally applied 5-HT from extracellular fluid (Fig. 3*B*). Interestingly, systemically administered fluvoxamine (10 mg/kg), which did not produce statistically significant effects in the TST, robustly inhibited clearance of intrahippocampally applied 5-HT. D-22 enhanced fluvoxamine-induced inhibition

of 5-HT clearance (one-way ANOVA, $F_{(3,27)} = 15.47$, $p < 0.05$) and of time spent immobile in the TST (one-way ANOVA, $F_{(3,31)} = 6.92$, $p < 0.05$). Thus our own data (Fig. 3*C*), and those of others (David et al., 2003), suggest a positive correlation between a drug's ability to inhibit 5-HT uptake and to produce antidepressant-like effects.

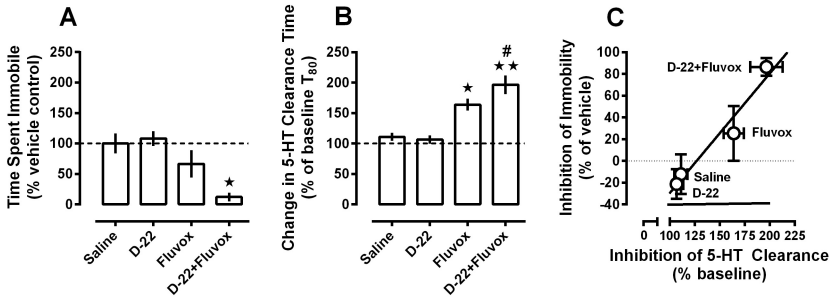

Figure 3. D-22 enhances the antidepressant-like and 5-HT clearance-inhibiting effects of fluvoxamine following systemic administration in wild-type C57BL/6 mice. ***A***, Doses of D-22 (0.1 mg/kg) and fluvoxamine (10 mg/kg), which by themselves did not significantly change time spent immobile in the TST compared with saline-injected control mice, markedly decreased immobility time when given together. Data are expressed as mean ± SEM percentage of time (s) immobile under saline control conditions (112 ± 18 s); $n = 7$–10 per treatment condition; one-way ANOVA, $F_{(3,31)} = 6.92$, $p < 0.05$; Newman–Keuls *post hoc* test, $^{*}p < 0.01$. ***B***, In a separate group of mice, using the same doses and injection-test intervals used for the TST, systemic administration of D-22 enhanced fluvoxamine-induced inhibition of 5-HT clearance in hippocampus. Data are expressed as a percentage of baseline T_{80} values (i.e., prevehicle or drug injection T_{80} clearance times for 5-HT) ± SEM; $n = 6$–9 per treatment condition; one-way ANOVA, $F_{(3,27)} = 15.47$, $p < 0.05$; Newman–Keuls *post hoc* test, $^{*}p < 0.01$, $^{**}p < 0.001$ versus saline; $\#p < 0.05$ versus fluvoxamine. ***C***, Regression analysis shows a strong, positive, correlation ($r^2 = 0.95$, $p = 0.025$) between the ability of a given treatment to inhibit 5-HT clearance in hippocampus and to produce antidepressant-like effects in the TST.

D-22 crosses the blood–brain barrier

The finding that both intrahippocampal and peripheral (i.p.) administration of D-22 produced similar inhibition of 5-HT clearance in hippocampus suggests that systemically administered D-22 probably exerts its effects on 5-HT clearance and behavior via central actions. To ensure that D-22 crossed the blood–brain barrier, we quantified D-22 in whole brains of three mice injected intraperitoneally with 10 mg/kg D-22 and three control mice injected with an equal volume of vehicle. Thirty minutes after the injection, the brain concentration of D-22 was 56 ± 27 pg/mg, confirming that D-22 reaches the brain following systemic administration.

D-22 enhances fluvoxamine-induced inhibition of 5-HT clearance without inducing elements of the serotonin syndrome

Drugs or drug combinations that produce excessive serotonergic activity can induce the serotonin syndrome (Sternbach, 1991; Kalueff et al., 2007). In humans, symptoms include hyperthermia, tachycardia, and rhabdomyolysis, which can result in death (Lane and Baldwin, 1997; Ener et al., 2003). Thus, an important consideration relating to our finding that D-22 enhances the ability of fluvoxamine to prevent 5-HT reuptake is that this drug combination conceivably could induce elements of the serotonin syndrome. In mice the serotonin syndrome consists of hindlimb abduction, low body posture, Straub tail, tremor, backward movement, head weaving, and forepaw treading (Fox et al., 2007). In the present study we treated mice with D-22 and fluvoxamine using the same doses and injection-test intervals as in the TST to examine whether this combination of D-22 and fluvoxamine, which produced maximal antidepressant-like activity, also induced elements of the serotonin syndrome. Different groups of mice were treated with either vehicle, 8-OH-DPAT (10 mg/kg i.p.), an agonist at 5-HT1A receptors that produces the serotonin syndrome in mice (Middlemiss and Fozard, 1983; Blackburn et al., 1984; Hensler and Truett, 1998; Fox et al., 2007), or 5-HTP (100 mg/kg i.p.), the precursor to 5-HT that also produces the serotonin syndrome in mice (Fox et al., 2007, 2008). Mice were then observed for 6 min periods, starting at 15, 30, 60, 120, and 240 min after the second injection. During each 6 min observation period, mice were scored at 1 min intervals for the presence or absence of serotonin syndrome elements, according to methods adapted from Fox et al., (2007). Results for the observation period starting 30 min after the second injection (i.e., the time corresponding to scoring in the TST) are shown in Table 1. As expected, the positive controls, 8-OH-DPAT and 5-HTP, produced hindlimb abduction and low body posture. In addition, 8-OH-DPAT produced Straub tail, tremor, and backward movement. Essentially none of these signs were observed in vehicle-treated animals. Importantly, the combination of D-22 and fluvoxamine also did not significantly produce any element of the serotonin syndrome, at any time during the 6 min test, 30 min following the second injection. Likewise, no elements of the serotonin syndrome were observed throughout the entire 4 h observation period following the combination of D-22 and fluvoxamine, suggesting that at these doses, the combination treatment does not produce side effects typically associated with excess activation of the serotonin system.

Enhancement by D-22 of fluvoxamine-induced antidepressant-like effects in the tail suspension test is attenuated in OCT3 knock-out mice

Our data show that D-22 can enhance the antidepressant-like activity of fluvoxamine. D-22 has affinity for OCT1, 2, and 3 subtypes as well as PMAT (Amphoux et al., 2006; Schömig et al., 2006; Koepsell et al., 2007; Duan and Wang, 2010). Our next studies therefore sought to gain insight into which of these transporters might be necessary for this action of D-22. OCT1 has limited ability to transport 5-HT and is not densely expressed in hippocampus (Amphoux et al., 2006; Schömig et al., 2006; Koepsell et al., 2007). OCT2 expression in brain is confined primarily to subventricular regions, though it is expressed in hippocampus (Wu et al., 1998, 1999; Amphoux et al., 2006; Bacq et al., 2012). OCT3 and PMAT are richly expressed in limbic regions, including hippocampus (Gründemann et al., 1998; Dahlin et al., 2007; Baganz et al., 2008; Gasser et al., 2009). Based on these reports and because our previous studies support a role for OCT3 in mediating the effects of D-22 on immobility time in the TST (Baganz et al., 2008, 2010), we turned to OCT3 KO mice to investigate the requirement of this transporter for D-22 to enhance the effects of fluvoxamine in the TST. Behavioral and neurochemical characterization of OCT3 KO mice has been reported previously (Vialou et al., 2008, Cui et al., 2009; Wultsch et al., 2009).

Using the same dosing and timing regimen as before, we found that like wild-type C57BL/6 mice, D-22 (0.1 mg/kg) and fluvoxamine (10 mg/kg), when given alone to OCT3 KO mice,

Table 1. D-22 together with fluvoxamine does not produce elements of the serotonin syndrome, unlike the 5-HT_{1A} receptor agonist 8-OH-DPAT and the 5-HT precursor, 5-HTP

Element of the 5-HT syndrome	Saline (Tween 80)	D-22 (0.1 mg/kg) + fluvoxamine (10 mg/kg)	8-OH-DPAT (10 mg/kg)	5-HTP (100 mg/kg)	$F[4,18]$	p value
Hindlimb abduction	0.2 ± 0.2 (2.0 ± 1.5)	0.2 ± 0.2	5.0 ± 1.0*	4.2 ± 1.1*	7.4	0.001
Low body posture	0 ± 0 (0 ± 0)	0 ± 0	4.4 ± 1.2*	4.2 ± 0.9*	10.7	0.001
Straub tail	0 ± 0 (0 ± 0)	0 ± 0	2.0 ± 0.9*	1.6 ± 0.6	4.4	0.012
Tremor	0 ± 0 (0 ± 0)	0 ± 0	1.6 ± 0.7*	0.4 ± 0.3	4.0	0.017
Backward movement	0 ± 0 (0 ± 0)	0.2 ± 0.2	1.0 ± 0.5*	0 ± 0	3.4	0.031
Head weaving	0 ± 0 (0 ± 0)	0 ± 0	0.6 ± 0.6	0 ± 0	0.9	0.495
Forepaw treading	0 ± 0 (0 ± 0)	0 ± 0	0.2 ± 0.2	0 ± 0	0.9	0.495

Data are expressed as mean ± SEM; $n = 5$ per treatment group, except 5% Tween 80 vehicle, $n = 3$; one-way ANOVA, followed by multiple comparisons with Newman–Keuls test. *Significantly different from saline, 5% Tween 80 vehicle and from D-22 + fluvoxamine.

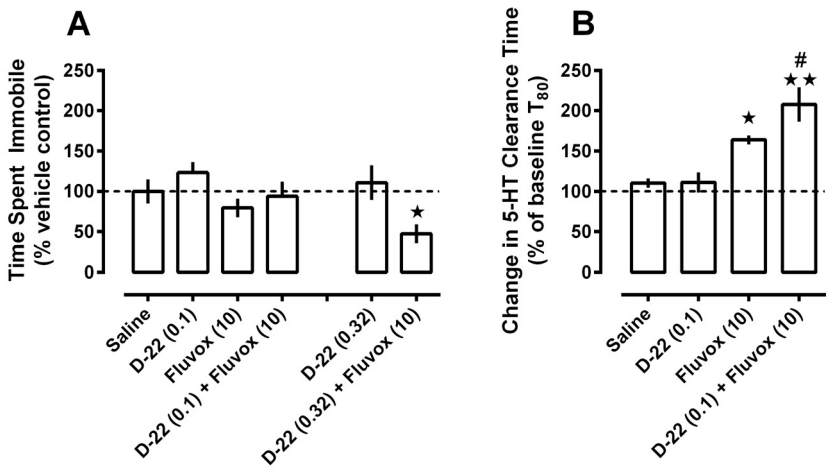

Figure 4. D-22 enhancement of the antidepressant-like effect of fluvoxamine in the TST is attenuated in OCT3 KO mice, whereas D-22 enhancement of the 5-HT clearance inhibiting effect fluvoxamine remains intact. ***A***, Doses of D-22 (0.1 and 0.32 mg/kg) and fluvoxamine (10 mg/kg) by themselves did not significantly change time spent immobile in the TST compared with saline-injected control mice. In contrast to wild-type mice (Fig. 3*A*), a dose of 0.1 mg/kg D-22 did not decrease immobility time when given together with fluvoxamine; however, a significant reduction in immobility time resulted when a higher dose of D-22 (0.32 mg/kg) was given with fluvoxamine. Data are expressed as mean ± SEM percentage of time (s) immobile under saline control conditions (127 ± 19 s); $n = 8–11$ per treatment condition; one-way ANOVA, $F_{(3,35)} = 3.17$, $p < 0.015$; Newman–Keuls *post hoc* test, $^*p < 0.05$, from saline and D-22 counterparts. ***B***, In a separate group of mice, using the injection-test intervals as for the TST, systemic administration of D-22 (0.1 mg/kg) enhanced fluvoxamine (10 mg/kg)-induced inhibition of 5-HT clearance in hippocampus. Data are expressed as a percentage of baseline T_{80} values (i.e., prevehicle or drug injection T_{80} clearance times for 5-HT) ± SEM; $n = 6–7$ per treatment condition; one-way ANOVA, $F_{(3,25)} = 12.34$, $p < 0.01$; Newman–Keuls *post hoc* test, $^*p < 0.05$, $^{**}p < 0.001$ versus saline; $\#p < 0.05$ versus fluvoxamine.

did not produce significant effects on time spent immobile compared with saline-treated mice. However, in contrast to wild-type mice, we found that D-22 failed to enhance the ability of fluvoxamine to reduce time spent immobile in the TST in OCT3 KO mice (Fig. 4*A*). These results suggest a key role for OCT3 in mediating this effect of D-22.

To confirm that this result was not due to reduced sensitivity of OCT3 KO mice to fluvoxamine, we performed dose–response studies and found that, like in wild-type mice, 10 mg/kg fluvoxamine was the largest dose that did not have significant effects by itself in the TST in OCT3 KO mice. Immobility time following vehicle injection in OCT3 KO mice was 127 ± 19 s ($n = 10$) (compared with 112 ± 18 s in wild-type mice, Fig. 3, legend). Similar to wild-type mice, fluvoxamine at doses of 3.2 and 10.0 mg/kg did not significantly influence time spent immobile in OCT3 KO mice (123 ± 14 s, $n = 8$, and 101 ± 15 s, $n = 11$, respectively), but significantly reduced immobility time at a dose of 32.0 mg/kg (47 ± 14 s, $n = 8$, one-way ANOVA, $F_{(3,33)} = 4.81$, $p < 0.05$). These data indicate that the inability of D-22 to enhance the antidepressant-like effect of fluvoxamine in the TST in OCT3 KO mice was not due to a reduction in sensitivity of these mice to fluvoxamine.

We next investigated if a higher dose of D-22 (0.32 mg/kg) would enhance the antidepressant-like effect of fluvoxamine (10 mg/kg). This dose of D-22 was the highest that did not decrease locomotion in wild-type mice. We found that this dose of D-22 did enhance the ability of fluvoxamine to reduce time spent immobile in OCT3 KO mice (Fig. 4*A*; one-way ANOVA, $F_{(3,35)} = 3.49$, $p < 0.05$); however, the magnitude of this enhancement was not as pronounced as that produced by the lower dose of D-22 in wild-type mice (Fig. 3*A*). In OCT3 KO mice given D-22 (0.32 mg/kg) together with fluvoxamine, time spent immobile decreased to 60 ± 15 s (47 ± 12% vehicle control, $n = 10$), whereas in wild-type mice D-22 (0.1 mg/kg) given in combination with fluvoxamine decreased immobility time essentially maximally (to 14 ± 8 s, or 12 ± 7% vehicle control, $n = 7$). An unpaired t test showed this difference to be significant ($t_{(15)} = 2.29$, $p < 0.05$). These data suggest that OCT3 plays a predominant role in mediating the ability of D-22 to enhance the antidepressant-like effect of fluvoxamine in the TST, but that D-22 can also produce this effect by actions at sites other than OCT3, albeit with lesser potency.

To examine whether the loss of antidepressant-like effect of the combination of D-22 (0.1 mg/kg) and fluvoxamine in the TST was associated with a reduced ability of the drug combination to inhibit 5-HT uptake in OCT3 KO mice, we used high-speed chronoamperometry to measure 5-HT clearance in the CA3 region of hippocampus under the same dose and injection-test interval as used previously. In contrast to the behavioral data, this drug combination inhibited 5-HT clearance in OCT3 KO mice similarly to that in wild-type mice (compare Figs. 3*B*, 4*B*). As we found in wild-type mice, D-22 (0.1 mg/kg) had no effect on 5-HT clearance, whereas fluvoxamine (10 mg/kg), which did not produce statistically significant effects in the TST, significantly inhibited clearance of intrahippocampally applied 5-HT in OCT3 KO mice. D-22 (0.1 mg/kg) enhanced fluvoxamine-induced inhibi-

tion of 5-HT clearance in OCT3 KO mice (one-way ANOVA, $F_{(3,25)} = 12.34$, $p < 0.05$).

Baseline (i.e., predrug) T_{80} values in OCT3 KO mice did not differ significantly ($p > 0.13$) among treatment groups and averaged 86 ± 6 s ($n = 26$), a value comparable to that obtained in wild-type mice (90 ± 4 s, $n = 69$). Likewise, baseline (i.e., predrug) signal amplitude values did not differ significantly ($p > 0.40$) among OCT3 KO mice treatment groups and averaged 0.63 ± 0.03 μM, comparable to that in wild-type mice (0.64 ± 0.02 μM).

Discussion

The major finding of the present studies is that the 5-HT clearance inhibiting and antidepressant-like effects of the SSRI fluvoxamine can be significantly augmented by coadministration of D-22. These data suggest that D-22-sensitive transporters may limit the ability of SSRIs to increase extracellular 5-HT and thus, limit their ability to produce antidepressant effects. D-22-sensitive transporters, such as OCTs and PMAT, may be useful targets for the development of new antidepressant medications to improve the therapeutic utility of SSRIs.

Implications for understanding the relationship between the ability of SSRIs to inhibit serotonin clearance and to produce antidepressant-like effects

An especially intriguing finding was the significant effect of the combination of fluvoxamine and D-22 to increase both the time course for 5-HT clearance and signal amplitude produced by 5-HT locally applied into the CA3 region of hippocampus. Previously, using similar approaches, we and others have not routinely observed an increase in amplitude of the 5-HT signal following administration of fluvoxamine. This has been a puzzling observation given that one would expect blockade of a transporter to increase both the clearance time and signal amplitude of exogenously applied neurotransmitter. Indeed, this is true for dopamine (DA) signals following blockade of the DA transporter (DAT) with drugs such as cocaine and nomifensine in DAT-rich regions, such as striatum and nucleus accumbens (Zahniser et al., 1999). This paradoxical lack of effect of SSRIs to increase 5-HT signal amplitude may well be attributed to the presence of D-22-sensitive transporters (OCTs and PMAT) and their expression level relative to SERT in hippocampus. As discussed earlier, these low-affinity, high-capacity transporters may become more effective at clearing 5-HT when extracellular 5-HT concentrations rise. Thus, following fluvoxamine, D-22-sensitive transporters may serve to prevent the 5-HT signal amplitude from increasing. Consistent with this notion is the observation that the effect of fluvoxamine on 5-HT clearance is most pronounced toward the tail of the signal, i.e., when the 5-HT concentration has fallen below the "reach" of low-affinity, high-capacity D-22-sensitive transporters (Fig. 1*A*). Thus, the combined effect of an SSRI and blocker of D-22-sensitive transporters is to not only further increase the duration that 5-HT remains in the extracellular fluid, but also to further increase the concentration (Daws et al., 2013).

Increased extracellular levels of 5-HT are considered to be an important trigger for ultimate therapeutic benefit in humans. Supporting this idea, our findings in wild-type mice show that antidepressant-like activity in the TST is positively correlated with the ability of a drug, or drug combination, to inhibit clearance of 5-HT in CA3 region of hippocampus (Fig. 3*C*). However, this was not the case in OCT3 KO mice, where the ability of D-22 to enhance the antidepressant-like effect of fluvoxamine in the TST was greatly attenuated, and yet inhibition of 5-HT clearance in the CA3 region of hippocampus was equivalent to that in wild-type mice. Thus, OCT3 appears to play a prominent role in mediating effects of D-22 in the TST, but does not appear to be involved in mediating the effect of D-22 to enhance fluvoxamine-induced inhibition of 5-HT clearance in the CA3 region of hippocampus. Together, these results indicate that antidepressant-like effects of a given treatment in the TST are not necessarily related to its ability to inhibit 5-HT clearance in the CA3 region of hippocampus. This raises the possibility that D-22's augmentation of the antidepressant-like effect of fluvoxamine in the TST is mediated by brain regions other than, or in addition to the CA3 region of hippocampus, and/or by its activity at other sites, possibly other OCT subtypes or PMAT, where it may inhibit uptake of 5-HT as well as other biogenic amines considered important in antidepressant-like response, discussed below.

Implications for increasing the clinical effects of antidepressants

The lack of symptom relief in many patients treated with currently available antidepressants emphasizes the need for novel pharmacological approaches with improved efficacy to treat depression. Our findings support D-22-sensitive uptake mechanisms as additional targets for the discovery of novel antidepressant therapies to maximize blockade of 5-HT uptake in brain. However, in addition to 5-HT, D-22-sensitive OCTs and PMAT are also low-affinity, high-capacity transporters for norepinephrine (NE) and DA (Wu et al., 1998; Amphoux et al., 2006; Cui et al., 2009; Bacq et al., 2012). Thus, while SSRIs have been the focus of this study, our results may generalize to other classes of antidepressants, including selective NE reuptake inhibitors, which block NE uptake via the NE transporter (NET), dual 5-HT-NE reuptake inhibitors, and triple-uptake inhibitors, which block transport of 5-HT, NE, and DA via their high-affinity transporters, SERT, NET, and DAT. To date, antidepressants that block SERT, NET, and DAT are thought to have the greatest clinical efficacy (Sulzer and Edwards, 2005; Chen and Skolnick, 2007). This may be in part attributed to the promiscuity that exists among SERT, NET, and DAT, which are all capable of low-affinity transport of their non-native biogenic amines (Pacholczyk et al., 1991; Daws et al., 1998; Norrholm et al., 2007; Rice and Cragg, 2008; Daws, 2009), as well as to the likelihood that successful treatment of depression requires targeting multiple neurotransmitter systems. Regardless, our findings suggest that the presence of D-22-sensitive transporters might also limit therapeutic efficacy of antidepressants other than SSRIs, and that blockade of D-22-sensitive transporters might serve to enhance their therapeutic efficacy by enhancing their ability to inhibit uptake of NE and DA, as well as 5-HT. Consistent with this idea, Hagan et al. (2011), using rotating disk voltammetry to measure 5-HT uptake into whole brain synaptosomes, showed that complete blockade of 5-HT uptake could only be achieved after incubating synaptosomes with blockers of SERT, NET, and DAT together with D-22. Thus, in terms of elevating extracellular levels of 5-HT and other biogenic amines to those that effectively treat depression, several lines of evidence, including the present study, point to blockade of D-22-sensitive transporters as a promising strategy.

In addition to being a potent blocker of low-affinity, high-capacity 5-HT transporters, D-22 has α-1-adrenoceptor antagonist properties (Russ et al., 1996). α-1 antagonism is unlikely to

mediate the enhancement by D-22 of fluvoxamine-induced inhibition of 5-HT clearance and antidepressant-like effects, because the α-1-adrenoceptor antagonist prazosin reportedly produces effects that are unlike those observed here with D-22. For example, prazosin decreases SSRI-induced increases of 5-HT levels (Rea et al., 2010), whereas D-22 enhanced the fluvoxamine-induced increase of 5-HT levels. In addition, prazosin increases immobility in the TST (Stone and Quartermain, 1999), whereas D-22 was inactive in this test when given alone at doses that enhanced effects of fluvoxamine. Finally, prazosin can block behavioral actions of antidepressants (Kostowski, 1985), whereas D-22 enhanced the antidepressant-like effects of fluvoxamine. Thus, it appears unlikely that α-1 antagonist properties underlie the ability of D-22 to enhance effects of fluvoxamine. Recently, the D-22 congener disprocynium-24 (D-24) has been reported to have antagonist properties not only at α-1 receptors, but also at α-2 receptors (Amphoux et al., 2010). It is presently unknown whether D-22 shares α-2 antagonist properties with D-24. If it does, these properties are unlikely to account for the effects of D-22 observed here, because the α-2 antagonist yohimbine does not affect 5-HT clearance (Ansah et al., 2003), and yohimbine increases immobility time in the TST (Ferrari et al., 1991). Together, there is evidence that D-22 has α-1-adrenoceptor antagonist properties, and evidence that α-1 antagonism produces effects that are opposite to those observed here with D-22. Thus, analogs of D-22 that lack α-1 antagonist properties may enhance effects of SSRIs even more markedly than D-22.

Although the idea of low-affinity, high-capacity transporters for 5-HT is not new, heralding back to the 1960s and 1970s when several groups reported promiscuous uptake of 5-HT by the so-called "uptake-2" transporter (Bertler et al., 1964; Fuxe et al., 1967; Lichtensteiger et al., 1967; Shaskan and Snyder, 1970), the identity of the transporter(s) has remained under some debate (Daws, 2009). In 2004, Schildkraut and Mooney proposed the extraneuronal monoamine transporter (uptake-2) as a target for development of more rapidly acting antidepressants, but still, the precise identity of "uptake-2" remained controversial. Since that time, several reports have emerged pointing to D-22-sensitive OCTs and PMAT as being primary uptake-2 (i.e., low-affinity, high-capacity) transporters for the biogenic amines (for review, see Daws, 2009). The challenge now will be to determine which of these might be the best to target for the discovery of new antidepressant drugs. While this field is still in its infancy OCT3 and more recently, OCT2, are emerging as leading candidates (Kitaichi et al., 2005; Baganz et al., 2008; Bacq et al., 2012). The markedly reduced potency of D-22 to enhance the antidepressant-like effect of fluvoxamine in the TST in OCT3 KO mice provides the first direct evidence that OCT3 is important for this action of D-22. Our behavioral and neurochemical results encourage further research into the role of D-22-sensitive transporters in regulating biogenic amine neurotransmission and behavior. Selective targeting of different OCT subtypes or PMAT may be a valid strategy to discover treatments with improved antidepressant efficacy, either as a cotreatment with existing antidepressant drugs or as novel 5-HT reuptake inhibitors with additional OCT or PMAT blocking properties.

References

Amphoux A, Vialou V, Drescher E, Brüss M, Mannoury La Cour C, Rochat C, Millan MJ, Giros B, Bönisch H, Gautron S (2006) Differential pharmacological in vitro properties of organic cation transporters and regional distribution in rat brain. Neuropharmacology 50:941–952. CrossRef Medline

Amphoux A, Millan MJ, Cordi A, Bönisch H, Vialou V, Mannoury la Cour C, Dupuis DS, Giros B, Gautron S (2010) Inhibitory and facilitory actions of isocyanine derivatives at human and rat organic cation transporters 1, 2 and 3: a comparison to human alpha 1- and alpha 2-adrenoceptor subtypes. Eur J Pharmacol 634:1–9. CrossRef Medline

Ansah TA, Ramamoorthy S, Montañez S, Daws LC, Blakely RD (2003) Calcium-dependent inhibition of synaptosomal serotonin transport by the alpha 2-adrenoceptor agonist 5-bromo-N-[4,5-dihydro-1H-imidazol-2-yl]-6-quinoxalinamine (UK14304). J Pharmacol Exp Ther 305:956–965. CrossRef Medline

Bacq A, Balasse L, Biala G, Guiard B, Gardier AM, Schinkel A, Louis F, Vialou V, Martres MP, Chevarin C, Hamon M, Giros B, Gautron S (2012) Organic cation transporter 2 controls brain norepinephrine and serotonin clearance and antidepressant response. Mol Psychiatry 17:926–939. CrossRef Medline

Baganz NL, Horton RE, Calderon AS, Owens WA, Munn JL, Watts LT, Koldzic-Zivanovic N, Jeske NA, Koek W, Toney GM, Daws LC (2008) Organic cation transporter 3: keeping the brake on extracellular serotonin in serotonin-transporter-deficient mice. Proc Natl Acad Sci U S A 105:18976–18981. CrossRef Medline

Baganz N, Horton R, Martin K, Holmes A, Daws LC (2010) Repeated swim impairs serotonin clearance via a corticosterone-sensitive mechanism: organic cation transporter 3, the smoking gun. J Neurosci 30:15185–15195. CrossRef Medline

Benmansour S, Cecchi M, Morilak DA, Gerhardt GA, Javors MA, Gould GG, Frazer A (1999) Effects of chronic antidepressant treatments on serotonin transporter function, density, and mRNA level. J Neurosci 19:10494–10501. Medline

Benmansour S, Owens WA, Cecchi M, Morilak DA, Frazer A (2002) Serotonin clearance in vivo is altered to a greater extent by antidepressant-induced downregulation of the serotonin transporter than by acute blockade of this transporter. J Neurosci 22:6766–6772. Medline

Benmansour S, Weaver RS, Barton AK, Adeniji OS, Frazer A (2012) Comparison of the effects of estradiol and progesterone on serotonergic function. Biol Psychiatry 71:633–641. CrossRef Medline

Bertler A, Falck B, Owman C (1964) Studies on 5-hydroxytryptamine stores in pineal gland of rat. Acta Physiol Scand [Suppl 239]:1–18. Medline

Blackburn TP, Kemp JD, Martin DA, Cox B (1984) Evidence that 5-HT agonist-induced rotational behaviour in the rat is mediated via 5-HT1 receptors. Psychopharmacology 83:163–165. CrossRef Medline

Blakely RD, Ramamoorthy S, Schroeter S, Qian Y, Apparsundaram S, Galli A, DeFelice LJ (1998) Regulated phosphorylation and trafficking of antidepressant-sensitive serotonin transporter proteins. Biol Psychiatry 44:169–178. CrossRef Medline

Campbell S, Macqueen G (2004) The role of the hippocampus in the pathophysiology of major depression. J Psychiatry Neurosci 29:417–426. Medline

Charney DS, Menkes DB, Heninger GR (1981) Receptor sensitivity and the mechanism of action of antidepressant treatment. implications for the etiology and therapy of depression. Arch Gen Psychiatry 38:1160–1180. CrossRef Medline

Chen Z, Skolnick P (2007) Triple uptake inhibitors: therapeutic potential in depression and beyond. Expert Opin Investig Drugs 16:1365–1377. CrossRef Medline

Cryan JF, Mombereau C, Vassout A (2005) The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. Neurosci Biobehav Rev 29:571–625. CrossRef Medline

Cui M, Aras R, Christian WV, Rappold PM, Hatwar M, Panza J, Jackson-Lewis V, Javitch JA, Ballatori N, Przedborski S, Tieu K (2009) The organic cation transporter-3 is a pivotal modulator of neurodegeneration in the nigrostriatal dopaminergic pathway. Proc Natl Acad Sci U S A 106:8043–8048. CrossRef Medline

Dahlin A, Xia L, Kong W, Hevner R, Wang J (2007) Expression and immunolocalization of the plasma membrane monoamine transporter in the brain. Neuroscience 146:1193–1211. CrossRef Medline

David DJ, Bourin M, Jego G, Przybylski C, Jolliet P, Gardier AM (2003) Effects of acute treatment with paroxetine, citalopram and venlafaxine *in vivo* on noradrenaline and serotonin outflow: a microdialysis study in Swiss mice. Br J Pharmacol 140:1128–1136. CrossRef Medline

Daws LC (2009) Unfaithful neurotransmitter transporters: focus on sero-

tonin uptake and implications for antidepressant efficacy. Pharmacol Ther 121:89–99. CrossRef Medline

Daws LC, Toney GM (2007) High-speed chronoamperometry to study kinetics and mechanisms for serotonin clearance in vivo. In: Electrochemical methods for neuroscience (Michael AC, Borland LM, eds). Boca Raton, FL: Taylor and Francis.

Daws LC, Toney GM, Gerhardt GA, Frazer A (1998) In vivo chronoamperometric measures of extracellular serotonin clearance in rat dorsal hippocampus: contribution of serotonin and norepinephrine transporters. J Pharmacol Exp Ther 286:967–976. Medline

Daws LC, Gould GG, Teicher SD, Gerhardt GA, Frazer A (2000) 5-HT(1B) receptor-mediated regulation of serotonin clearance in rat hippocampus in vivo. J Neurochem 75:2113–2122. Medline

Daws LC, Moñtanez S, Owens WA, Gould GG, Frazer A, Toney GM, Gerhardt GA (2005) Transport mechanisms governing serotonin clearance in vivo revealed by high-speed chronoamperometry. J Neurosci Methods 143:49–62. CrossRef Medline

Daws LC, Koek W, Mitchell NC (2013) Revisiting serotonin reuptake inhibitors and the therapeutic potential of "uptake-2" in psychiatric disorders. ACS Chem Neurosci 4:16–21. CrossRef Medline

Duan H, Wang J (2010) Selective transport of monoamine neurotransmitters by human plasma membrane monoamine transporter and organic cation transporter 3. J Pharmacol Exp Ther 335:743–753. CrossRef Medline

Ener RA, Meglathery SB, Van Decker WA, Gallagher RM (2003) Serotonin syndrome and other serotonergic disorders. Pain Med 4:63–74. CrossRef Medline

Engel K, Wang J (2005) Interaction of organic cations with a newly identified plasma membrane monoamine transporter. Mol Pharmacol 68:1397–1407. CrossRef Medline

Engel K, Zhou M, Wang J (2004) Identification and characterization of a novel monoamine transporter in the human brain. J Biol Chem 279:50042–50049. CrossRef Medline

Ferrari F, Cassinadri M, Tartoni PL, Tampieri A (1991) Effects of B-HT 920 in the tail-suspension test. Pharmacol Res 24:75–81. CrossRef Medline

Fox MA, Jensen CL, Gallagher PS, Murphy DL (2007) Receptor mediation of exaggerated responses to serotonin-enhancing drugs in serotonin transporter (SERT)-deficient mice. Neuropharmacology 53:643–656. CrossRef Medline

Fox MA, Jensen CL, French HT, Stein AR, Huang SJ, Tolliver TJ, Murphy DL (2008) Neurochemical, behavioral, and physiological effects of pharmacologically enhanced serotonin levels in serotonin transporter (SERT)-deficient mice. Psychopharmacology 201:203–218. CrossRef Medline

Franklin K, Paxinos G (1997) The mouse brain in stereotaxic coordinates. Sydney: Academic.

Fujishiro J, Imanishi T, Onozawa K, Tsushima M (2002) Comparison of the anticholinergic effects of the serotonergic antidepressants, paroxetine, fluvoxamine and clomipramine. Eur J Pharmacol 454:183–188. CrossRef Medline

Fuxe K, Hokfelt T, Ungerstedt U (1967) Studies on uptake mechanisms in central monoamine neurones. Acta Pharmacol Toxicol 25:[Suppl 4]:8. Medline

Gasser PJ, Lowry CA, Orchinik M (2006) Corticosterone-sensitive monoamine transport in the rat dorsomedial hypothalamus: potential role for organic cation transporter 3 in stress-induced modulation of monoaminergic neurotransmission. J Neurosci 26:8758–8766. CrossRef Medline

Gasser PJ, Orchinik M, Raju I, Lowry CA (2009) Distribution of organic cation transporter 3, a corticosterone-sensitive monoamine transporter, in the rat brain. J Comp Neurol 512:529–555. CrossRef Medline

Gerhardt GA (1995) Neuromethods: voltammetric methods in brain systems. In: Rapid chronocoulometric measurements of norepinephrine overflow and clearance in CNS tissues, Vol 27 (Boulton AA., Baker GB, Adams RN, eds), pp 117–151. Totowa, NJ: Humana.

Gründemann D, Köster S, Kiefer N, Breidert T, Engelhardt M, Spitzenberger F, Obermüller N, Schömig E (1998) Transport of monoamine transmitters by the organic cation transporter type 2, OCT2. J Biol Chem 273:30915–30920. CrossRef Medline

Hagan CE, Schenk JO, Neumaier JF (2011) The contribution of low-affinity transport mechanisms to serotonin clearance in synaptosomes. Synapse 65:1015–1023. CrossRef Medline

Hensler JG, Truett KA (1998) Effect of chronic serotonin-2 receptor agonist or antagonist administration on serotonin-1A receptor sensitivity. Neuropsychopharmacology 19:354–364. CrossRef Medline

Kalueff AV, Fox MA, Gallagher PS, Murphy DL (2007) Hypolocomotion, anxiety and serotonin syndrome-like behavior contribute to the complex phenotype of serotonin transporter knockout mice. Genes Brain Behav 6:389–400. CrossRef Medline

Kirsch I, Deacon BJ, Huedo-Medina TB, Scoboria A, Moore TJ, Johnson BT (2008) Initial severity and antidepressant benefits: a meta-analysis of data submitted to the food and drug administration. PLoS Med 5:e45. CrossRef Medline

Kitaichi K, Fukuda M, Nakayama H, Aoyama N, Ito Y, Fujimoto Y, Takagi K, Takagi K, Hasegawa T (2005) Behavioral changes following antisense oligonucleotide-induced reduction of organic cation transporter-3 in mice. Neurosci Lett 382:195–200. CrossRef Medline

Koepsell H, Lips K, Volk C (2007) Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. Pharm Res 24:1227–1251. CrossRef Medline

Kostowski W (1985) Possible relationship of the locus coeruleus–hippocampal noradrenergic neurons to depression and mode of action of antidepressant drugs. Pol J Pharmacol Pharm 37:727–743. Medline

Lamhonwah AM, Hawkins CE, Tam C, Wong J, Mai L, Tein I (2008) Expression patterns of the organic cation/carnitine transporter family in adult murine brain. Brain Dev 30:31–42. CrossRef Medline

Lane R, Baldwin D (1997) Selective serotonin reuptake inhibitor-induced serotonin syndrome: review. J Clin Psychopharmacol 17:208–221. CrossRef Medline

Lichtensteiger W, Mutzner U, Langemann H (1967) Uptake of 5-hydroxytryptamine and 5-hydroxytryptophan by neurons of the central nervous system normally containing catecholamines. J Neurochem 14:489–497. CrossRef Medline

Middlemiss DN, Fozard JR (1983) 8-hydroxy-2-(di-n-propylamino)-tetralin discriminates between subtypes of the 5-HT1 recognition site. Eur J Pharmacol 90:151–153. CrossRef Medline

Montañez S, Owens WA, Gould GG, Murphy DL, Daws LC (2003) Exaggerated effect of fluvoxamine in heterozygote serotonin transporter knockout mice. J Neurochem 86:210–219. Medline

Norrholm SD, Horton DB, Dwoskin LP (2007) The promiscuity of the dopamine transporter: implications for the kinetic analysis of [^{3}H]serotonin uptake in rat hippocampal and striatal synaptosomes. Neuropharmacology 53:982–989. CrossRef Medline

Pacholczyk T, Blakely RD, Amara SG (1991) Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. Nature 350:350–354. CrossRef Medline

Perez XA, Andrews AM (2005) Chronoamperometry to determine differential reductions in uptake in brain synaptosomes from serotonin transporter knockout mice. Anal Chem 77:818–826. CrossRef Medline

Rea K, Folgering J, Westerink BH, Cremers TI (2010) Alpha1-adrenoceptors modulate citalopram-induced serotonin release. Neuropharmacology 58:962–971. CrossRef Medline

Rice ME, Cragg SJ (2008) Dopamine spillover after quantal release: rethinking dopamine transmission in the nigrostriatal pathway. Brain Res Rev 58:303–313. CrossRef Medline

Russ H, Friedgen B, Königs B, Schumacher C, Graefe KH, Schömig E (1996) Pharmacokinetic and alpha 1-adrenoceptor antagonistic properties of two cyanine-type inhibitors of extraneuronal monoamine transport. Naunyn Schmiedebergs Arch Pharmacol 354:268–274. Medline

Schildkraut JJ, Mooney JJ (2004) Toward a rapidly acting antidepressant: the normetanephrine and extraneuronal monoamine transporter (uptake 2) hypothesis. Am J Psychiatry 161:909–911. CrossRef Medline

Schömig E, Babin-Ebell J, Russ H (1993) 1,1′-Diethyl-2,2′-cyanine (decynium22) potently inhibits the renal transport of organic cations. Naunyn Schmiedebergs Arch Pharmacol 347:379–383. CrossRef Medline

Schömig E, Lazar A, Gründemann D (2006) Extraneuronal monoamine transporter and organic cation transporters 1 and 2: a review of transport efficiency. Handb Exp Pharmacol 175:151–180. CrossRef Medline

Shaskan EG, Snyder SH (1970) Kinetics of serotonin accumulation into slices from rat brain: relationship to catecholamine uptake. J Pharmacol Exp Ther 175:404–418. Medline

Sternbach H (1991) The serotonin syndrome. Am J Psychiatry 148:705–713. Medline

Steru L, Chermat R, Thierry B, Simon P (1985) The tail suspension test: a

new method for screening antidepressants in mice. Psychopharmacology 85:367–370. CrossRef Medline

Stone EA, Quartermain D (1999) Alpha-1-noradrenergic neurotransmission, corticosterone, and behavioral depression. Biol Psychiatry 46:1287–1300. CrossRef Medline

Sulzer D, Edwards RH (2005) Antidepressants and the monoamine masquerade. Neuron 46:1–2. CrossRef Medline

Vialou V, Balasse L, Dumas S, Giros B, Gautron S (2007) Neurochemical characterization of pathways expressing plasma membrane monoamine transporter in the rat brain. Neuroscience 144:616–622. CrossRef Medline

Vialou V, Balasse L, Callebert J, Launay JM, Giros B, Gautron S (2008) Altered aminergic neurotransmission in the brain of organic cation transporter 3-deficient mice. J Neurochem 106:1471–1482. Medline

Williams JM, Owens WA, Turner GH, Saunders C, Dipace C, Blakely RD, France CP, Gore JC, Daws LC, Avison MJ, Galli A (2007) Hypoinsulinemia regulates amphetamine-induced reverse transport of dopamine. PLoS Biol 5:e274. CrossRef Medline

Wu X, Kekuda R, Huang W, Fei YJ, Leibach FH, Chen J, Conway SJ, Ganapathy V (1998) Identity of the organic cation transporter OCT3 as the extraneuronal monoamine transporter (uptake2) and evidence for the expression of the transporter in the brain. J Biol Chem 273:32776–32786. CrossRef Medline

Wu X, Huang W, Prasad PD, Seth P, Rajan DP, Leibach FH, Chen J, Conway SJ, Ganapathy V (1999) Functional characteristics and tissue distribution pattern of organic cation transporter 2 (OCTN2) an organic cation/carnitine transporter. J Pharmacol Exp Ther 290:1482–1492. Medline

Wultsch T, Grimberg G, Schmitt A, Painsipp E, Wetzstein H, Breitenkamp AF, Gründemann D, Schömig E, Lesch KP, Gerlach M, Reif A (2009) Decreased anxiety in mice lacking organic cation transporter 3. J Neural Transm 116:689–697. CrossRef Medline

Zahniser NR, Larson GA, Gerhardt GA (1999) In vivo dopamine clearance rate in rat striatum: regulation by extracellular dopamine concentration and dopamine transporter inhibitors. J Pharmacol Exp Ther 289:266–277. Medline

Zwart R, Verhaagh S, Buitelaar M, Popp-Snijders C, Barlow DP (2001) Impaired activity of the extraneuronal monoamine transporter systems known as uptake-2 in Ort3/Slc22a3-deficient mice. Mol Cell Biol 21:4188–4196. CrossRef Medline

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Antidepressant-like drug effects in juvenile and adolescent mice in the tail suspension test: relationship with hippocampal serotonin and norepinephrine transporter expression and function

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Depression is a major health problem for which most patients are not effectively treated. This problem is further compounded in children and adolescents where only two antidepressants [both selective serotonin reuptake inhibitors (SSRIs)] are currently approved for clinical use. Mouse models provide tools to identify mechanisms that might account for poor treatment response to antidepressants. However, there are few studies in adolescent mice and none in juvenile mice. The tail suspension test (TST) is commonly used to assay for antidepressant-like effects of drugs in adult mice. Here we show that the TST can also be used to assay antidepressant-like effects of drugs in C57Bl/6 mice aged 21 (juvenile) and 28 (adolescent) days post-partum (P). We found that the magnitude of antidepressant-like response to the SSRI escitalopram was less in P21 mice than in P28 or adult mice. The smaller antidepressant response of juveniles was not related to either maximal binding (B_{max}) or affinity (K_d) for [^{3}H]citalopram binding to the serotonin transporter (SERT) in hippocampus, which did not vary significantly among ages. Magnitude of antidepressant-like response to the tricyclic desipramine was similar among ages, as were B_{max} and K_d values for [^{3}H]nisoxetine binding to the norepinephrine transporter in hippocampus. Together, these findings suggest that juvenile mice are less responsive to the antidepressant-like effects of escitalopram than adults, but that this effect is not due to delayed maturation of SERT in hippocampus. Showing that the TST is a relevant behavioral assay of antidepressant-like activity in juvenile and adolescent mice sets the stage for future studies of the mechanisms underlying the antidepressant response in these young populations.

Keywords: antidepressant, selective serotonin reuptake inhibitor, tricyclic, serotonin transporter, norepinephrine transporter, juvenile, adolescent, depression

INTRODUCTION

Depression is a major public health problem for which most patients are not effectively treated. This problem is further compounded in children and adolescents by limited pharmacological treatment options (Bylund and Reed, 2007). The selective serotonin reuptake inhibitor (SSRI) fluoxetine is currently the only FDA approved treatment for depression in children and adolescents up to 18 years old, and escitalopram is approved for children and adolescents age 12 and older. Exacerbating the situation further, children and adolescents respond poorly to these antidepressants compared with adults (Tsapakis et al., 2008; Hetrick et al., 2009, 2010). Given the high prevalence of adolescent depression, affecting 4–8% of the population with an incidence of 25% by the end of adolescence (Kessler et al., 2001; Bujoreanu et al., 2011) and, of major concern, the high prevalence of suicide in this young population (the third leading cause of death in the 15- to 19-year age group; Reed et al., 2008), there is a clear need to understand the neural mechanisms accounting for these differences between children and adolescents on the one hand and adults on the other, with the hope to uncover targets for the development of more effective treatments. However, despite many reports showing marked differences in the antidepressant response of children and adolescents compared with adults (Bylund and Reed, 2007; Tsapakis et al., 2008; Hetrick et al., 2009, 2010; Hazell and Mirzaie, 2013) there is a paucity of studies investigating the underlying mechanisms. Thus, reasons for the age-dependency of antidepressant response remain poorly understood.

Animal models are needed to examine the mechanisms underlying age-dependent effects of antidepressants. To date, there are only a few preclinical studies of antidepressants in juvenile and early adolescent animals, and most have been conducted using rats. SSRIs were found to reduce time spent immobile in the forced swim test (FST), an index of antidepressant-like activity, in rats as young as postnatal day (P) 21 as well as in adults, whereas blockers

of the norepinephrine (NE) transporter, such as the tricyclic antidepressant desipramine (DMI), were ineffective in the FST in rats younger than P28 (Pechnick et al., 2008; Reed et al., 2008). The mechanistic basis for these findings remains to be determined, but is thought to involve the delayed maturation of the NE neurotransmitter system relative to the serotonin (5-HT) system. In terms of the actual drug targets themselves, i.e., the serotonin and norepinephrine transporters (SERT and NET, respectively), information about their expression in juvenile and adolescent animals is sparse. Using quantitative autoradiography, Galineau et al. (2004) reported a triphasic profile for SERT in amygdala and hypothalamus of rats where expression peaked around P21, decreased at P28 and plateaued through P70, the oldest age tested (see also Daws and Gould, 2011). For NET, Sanders et al. (2005) also using autoradiography, reported that expression of NET in some brain regions (e.g., locus coeruleus) was much greater in rats aged P20 than in adults, while in other regions NET expression in P20 rats was either less than (e.g., CA3 region of hippocampus) or similar (e.g., cortex, CA1 and CA2 regions of hippocampus, dentate gyrus) to that of adult rats. Thus, it is not clear from studies in rats, if expression or activity of SERT and NET correlates positively with the emergence of an antidepressant-like response to SSRIs and NET blockers.

Lacking are studies in mice to probe the mechanistic basis underlying differences in antidepressant-efficacy among juveniles, adolescents, and adults. The relative ease with which mice can be genetically manipulated makes them a powerful tool for preclinical research. However, there are few studies that have used adolescent (≥P28) mice to investigate antidepressant-like response (Bourin et al., 1998; David et al., 2001a; Mason et al., 2009) and none that have used mice younger than P28. Although mice have been used to examine the consequences of antidepressant treatment during prenatal, early postnatal and adult periods, juvenile and adolescent periods remain largely unexplored. Likewise, little is known about SERT and NET expression during these juvenile and adolescent periods in mice.

The tail suspension test (TST) is a preclinical test with good predictive validity that has become one of the most widely used models for assessing antidepressant-like activity in adult mice (Cryan et al., 2005). Currently there is only one report of its use in adolescent (P35) mice. Thus, it is unknown if the TST can be used to detect antidepressant-like effects of drugs in early adolescent (P28) and juvenile (P21) mice. The goals of the present study were twofold: first, to examine if the TST can be used to measure antidepressant-like activity in P21 and P28 mice; and second, to begin to examine the relationship between antidepressant-like activity and the expression and affinity of hippocampal SERT and NET in juvenile, adolescent, and adult mice.

MATERIALS AND METHODS

ANIMALS

Juvenile (P21), early adolescent (P28), and adult (P62–90) male and female C57Bl/6 mice were obtained from an in house breeding colony (breeding pairs originally obtained from Jackson Lab). Body weights for male mice ranged from 6.6 to 9.8 g for P21, from 9.7 to 18.5 g for P28, and from 23.7 to 42.8 g for adults, and body weights for female mice ranged from 6.3 to 10.0 g for P21, from 10.8 to 15.1 g for P28, and from 19.1 to 31.3 g for adults. Animals were housed in a temperature-controlled (24°C) vivarium maintained on a 14/10-h light/dark cycle (lights on at 07:00, experiments conducted during the light period) in plastic cages (29 cm × 18 cm × 13 cm) containing rodent bedding (Sani-chips, Harlan Teklad, Madison, WI, USA) with free access to food (Rodent sterilizable diet, Harlan Teklad, Madison, WI, USA) and water. After weaning on postnatal day 21, mice were housed in groups of five with same-sex peers. All procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council 1996), and with the Institutional Animal Care and Use Committee, The University of Texas Health Science Center at San Antonio.

TAIL SUSPENSION TEST

The TST was conducted based on the original description by Steru et al. (1985) [for a review, see (Castagne et al., 2011)]. On the day before testing, mice were moved from the colony room and housed overnight in a holding room adjoining the procedure room. On the test day, mice were placed in the procedure room and allowed 1–2 h to acclimate before receiving an injection of saline vehicle (subcutaneously [sc] or intraperitoneally [ip]), escitalopram (10 mg/kg, sc), or DMI (32 mg/kg, ip). Routes of drug administration were based on results in adult mice reported by Cryan et al. (2005) and Sanchez et al. (2003). Each mouse was tested only once (i.e., not given multiple drugs nor exposed to the TST on multiple occasions). Drugs or saline were injected 30 min before testing. Immediately before testing, the distal end of the tail was fastened to a flat aluminum (2 × 0.3 × 10 cm) bar using adhesive tape at a 90° angle to the longitudinal axis of the mouse tail and the aluminum bar, with a distance of 3–4 cm between the base of the tail and the edge of the bar. A hole opposite the taped end of the bar was used to secure the bar to a hook in the ceiling of a visually isolated white test box (40 cm × 40 cm × 40 cm). Each mouse was suspended by its tail for 6 min, allowing the ventral surface and front and hind limbs to be video-recorded using a digital camera facing the test box. Total time immobile was measured (in seconds) during the entire 6 min test period. Immobility was defined as the absence of initiated movements, and included passive swaying of the body. A mouse was excluded from the experiments if it climbed and held on to its tail or the aluminum bar for a period of 3 s or longer. In the present study no mice aged P21 or P28 were excluded. Approximately 5% of adult mice were excluded. Immobility was scored manually by observers watching the video and who were blind to the treatment. Typically two observers scored each videotape with excellent inter-observer agreement ($r^2 > 0.9$).

Initial experiments were designed to examine the utility of the TST in juvenile and adolescent mice. Male and female mice were given either a sc or ip injection of saline, corresponding to the route of administration for escitalopram and DMI, respectively. The purpose of these initial experiments was to identify possible effects of age, gender, and route of administration, as well as any interactions among these factors, on immobility in the TST. Age affected basal immobility, and did so in a similar manner in both

genders after both routes of administration (see Results). Subsequent experiments investigated the two reference antidepressant drugs, escitalopram (10 mg/kg, sc) and DMI (32 mg/kg, ip), in male mice. Drug doses were selected based on preliminary data obtained in our laboratory that showed these doses to be the lowest to produce maximal effects on immobility in adult C57Bl/6J mice.

[^{3}H]CITALOPRAM AND [^{3}H]NISOXETINE SATURATION BINDING IN HIPPOCAMPAL HOMOGENATES

All binding experiments were carried out using tissue from male C57Bl/6 mice.

[^{3}H]citalopram binding to SERT

Saturation binding of [^{3}H]citalopram in membrane homogenate preparations from mouse hippocampi was carried out following the methods of D'Amato et al. (1987) with minor modifications. Briefly, male mice were decapitated, the brain removed and hippocampi collected. Hippocampi from individual mice were homogenized in 25 ml of 4°C 50 mM Tris, 120 mM NaCl, 5 mM KCl buffer (pH 7.4 at 25°C), at 2600 rpm on a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY, USA). The homogenate was centrifuged for 10 min at 30,600 × *g* at 4°C. The supernatant was discarded, and the pellet re-suspended on ice using a Potter Elvehjem glass and Teflon homogenizer in 25 ml ice-cold buffer. The homogenate was re-centrifuged for 10 min at 30,600 × *g*. The final pellet was re-suspended to yield a protein concentration of approximately 0.5–1.2 μg/μl. Protein was quantified spectrophotometrically on a plate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) using Bradford reagent (Sigma, St. Louis, MO, USA). Binding assays were run in triplicate for each hippocampal membrane homogenate preparation. Homogenates were incubated at 25°C for 1 h in buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl) containing 0.1–10 nM [^{3}H]citalopram (Perkin Elmer). Non-specific binding was defined by addition of 10 μM sertraline (Pfizer). Incubation was terminated by addition of 4 ml of ice cold buffer and rapid filtration under vacuum onto Whatman GF/B filter paper strips (Brandel, Gaithersburg, MD, USA) pre-soaked in 5% polyethyleneimine (Sigma). Filters were washed twice and radioactivity trapped on the filters was measured by liquid scintillation counting using a Beckman 6500 (Beckman, Brea, CA, USA) with efficiencies of 40–65%. Binding data were analyzed by non-linear regression using GraphPad Prism 5.04.

[^{3}H]nisoxetine binding to NET

Saturation binding of [^{3}H]nisoxetine in membrane homogenate preparations from mouse hippocampi was carried out following the methods of Tejani-Butt (1992). Hippocampal homogenate preparation for [^{3}H]nisoxetine binding was as described for [^{3}H] citalopram binding, except that hippocampi were pooled from two mice to yield protein concentrations of 1.0–1.7 μg/μl, the buffer was pH 7.4 at 4°C and the final washed pellet was re-suspended in 50 mM Tris, 300 mM NaCl, 5 mM KCl (pH 7.4 at 4°C). Binding assays were carried out for 4 h at 4°C in 50 mM Tris, 300 mM NaCl, 5 mM KCl at the same volumes used for [^{3}H] citalopram binding. [^{3}H] nisoxetine concentration ranged from 0.5 to 30 nM for the saturation assays. Non-specific binding was defined with 10 μM mazindol (Sigma, St Louis, MO, USA). Data collection and analysis were the same as described for [^{3}H]citalopram binding.

STATISTICAL ANALYSIS

Statistical analyses were performed using Prism 5.04 (GraphPad, San Diego, CA, USA) and NCSS 2007 (Kaysville, UT, USA). TST data were analyzed using ANOVA, followed by Tukey's multiple comparison tests. Binding data were analyzed using Kruskal–Wallis test because of significant differences in standard deviations among age groups (Bartlett's test). All data are expressed as mean ± standard error of the mean (SEM), and $P < 0.05$ was considered statistically significant.

DRUGS

Escitalopram oxalate [Shanco International Inc. (Hazlet, NJ, USA)] and DMI hydrochloride [Sigma-Aldrich (St. Louis, MO, USA)] were dissolved in physiological saline. Escitalopram was injected sc at doses expressed as base per kilogram body weight (Sanchez et al., 2003). DMI was injected ip at doses expressed as salt per kilogram body weight. The injection volume was 10 ml/kg.

RESULTS

USE OF THE TST IN JUVENILE AND ADOLESCENT MICE

The TST is a preclinical test with good predictive validity that is widely used to detect antidepressant-like activity (Cryan and Holmes, 2005; Cryan et al., 2005), and that has been used in mice as young as P35 (Mason et al., 2009). Antidepressant-like activity in this test is defined by the ability of a drug to reduce the time a mouse spends immobile. We first examined if the TST could be used in P21 and P28 mice, given the possibility that young mice may display so little baseline immobility that the effect of a drug to reduce immobility further may not be detectible. To this end, six separate groups of mice (male and female, $n = 20$ of each gender and each age, P21, P28, or adult) received an injection of saline either sc or ip ($n = 10$ of each gender and age receiving saline sc, and $n = 10$ of each gender and age receiving saline ip) and time spent immobile in a 6 min test was quantified. There was no significant effect of gender or route of administration on immobility time, a finding that is consistent with reports for adult mice (e.g., David et al., 2001b; Jones and Lucki, 2005; Andreasen and Redrobe, 2009). There was however, a significant effect of age [$F(2, 119) = 6.63$, $P < 0.0025$]. Because there were no significant interactions among age, gender, and route of administration, data were collapsed with age as the only variable (**Figure 1**). P21 mice spent significantly less time immobile (99 ± 8 s) than P28 (123 ± 7 s) or adult mice (135 ± 6 s), and P28 mice did not differ significantly from adults. A factor that might contribute to reduced immobility time in P21 mice is their smaller size. Regression analyses of immobility time as a function of body weight for all ages and both genders, inclusive, revealed an overall positive correlation ($r = 0.24$, $P < 0.01$). However, regression analyses within each age group revealed no significant correlation between body weight and immobility time (P21, $r = -0.12$, $P = 0.46$; P28, $r = 0.23$, $P = 0.11$; adult, $r = 0.05$, $P = 0.66$, data not shown).

These data show that juvenile and adolescent mice spend sufficient time immobile in the TST that detection of a drug effect

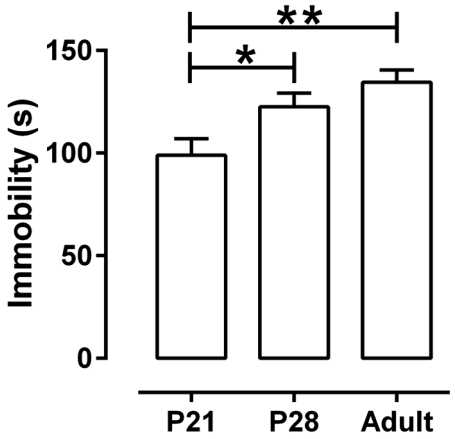

FIGURE 1 | Immobility time in the TST as a function of age. Juvenile mice (P21) spent significantly less time immobile than either adolescent (P28) or adult mice ($P > 62$). Each age group consisted of 40 animals treated with saline (20 males and 20 females, half of them treated sc and the other half ip). Because there were neither main nor interaction effects of gender and route of administration, data are shown collapsed with age as the only factor. $*P < 0.05$, $**P < 0.01$ (Tukey *post hoc* test). Data are mean and SEM.

to decrease immobility should be possible. To test this, juvenile and adolescent mice were treated acutely with either escitalopram (sc) or DMI (ip), two antidepressants known to produce robust effects in the TST in adult mice (Sanchez et al., 2003; O'Leary et al., 2007).

REFERENCE ANTIDEPRESSANTS REDUCE IMMOBILITY ACROSS AGES

Escitalopram (10 mg/kg, sc) and DMI (32 mg/kg, ip) reduced immobility time in the TST in all age groups [$F(1, 51) = 66.45$, $P < 0.01$] (**Figure 2**). However, the extent to which they decreased immobility differed among the age groups. For escitalopram there was a significant interaction between treatment and age [$F(2, 51) = 5.08$, $P < 0.01$] because escitalopram reduced immobility less in P21 mice than in P28 and adult mice. For DMI there was a significant effect of age [$F(2, 51) = 7.14$, $P < 0.01$] and DMI tended to reduce immobility more in P21 and P28 mice than in adults, but the interaction between treatment and age did not reach statistical significance. Sample sizes for saline-, escitalopram-, and DMI-treated mice, respectively, were 20, 8, and 10 for mice aged P21; 20, 8, and 8 for mice aged P28, and 20, 10, and 9 for adult mice. The larger sample size for saline-treated mice is due to pooling data from male mice injected with saline sc ($n = 10$) and ip ($n = 10$) for each age. These results show that the TST can be used to examine antidepressant-like drug effects in mice as young as P21. Next we investigated the relationship between the antidepressant-like effects of escitalopram and DMI and the expression of their targets, the SERT and NET, respectively, in hippocampus of P21, P28, and adult mice.

[^{3}H]CITALOPRAM AND [^{3}H]NISOXETINE SATURATION BINDING IN HIPPOCAMPUS AS A FUNCTION OF AGE

As shown in **Figures 3A,C,E** and **Table 1**, [^{3}H]citalopram saturation binding in mouse hippocampal homogenates revealed

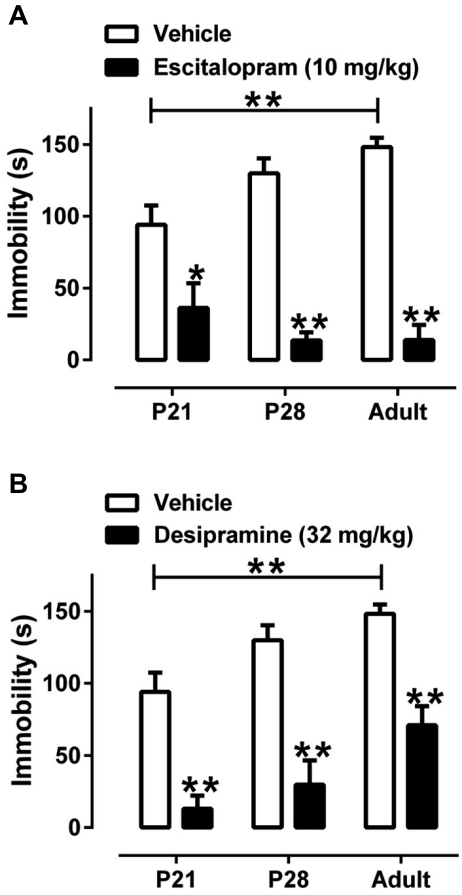

FIGURE 2 | Effect of escitalopram and DMI to reduce time spent immobile in the TST in male juvenile, adolescent, and adult mice. **(A)** Escitalopram (10 mg/kg, sc) significantly reduced immobility time in all age groups. There was a significant interaction between age and treatment revealing that escitalopram is less effective in reducing immobility time in P21 mice, compared with P28 or adult mice. **(B)** DMI (32 mg/kg, ip) significantly reduced immobility time in all age groups. Sample sizes were as follows: P21, $n = 38$ (20 saline, 8 escitalopram, 10 DMI); P28, $n = 37$ (20 saline, 8 escitalopram, 8 DMI); adult mice $n = 39$ (20 saline, 10 escitalopram, 9 DMI). There was no main effect of route of saline administration, so saline data were collapsed with age and drug as the only factors; two-way ANOVA with Tukey's *post hoc* tests, $*P < 0.05$, $**P < 0.01$. Data are mean and SEM.

no significant difference in maximal binding (B_{max}) or affinity (K_d) values among P21 ($n = 10$), P28 ($n = 8$), and adult ($n = 9$) male mice. Likewise, [^{3}H]nisoxetine saturation binding in mouse hippocampal homogenates revealed no significant difference in B_{max} or K_d values among P21 ($n = 9$), P28 ($n = 9$), and adult ($n = 7$) male mice (**Figures 3B,D,F**; **Table 1**). Of note is the greater variability in K_d values for [^{3}H]citalopram binding in P21 mice, compared with their P28 and adult counterparts ($P < 0.001$, Bartlett's test). Similarly, the variance of the K_d values for [^{3}H]nisoxetine binding in young mice was greater than in adults ($P < 0.01$, Bartlett's test), suggesting that these young ages may represent a transitional period where SERT and NET are shifting toward the functional activity state of adult SERT and NET.

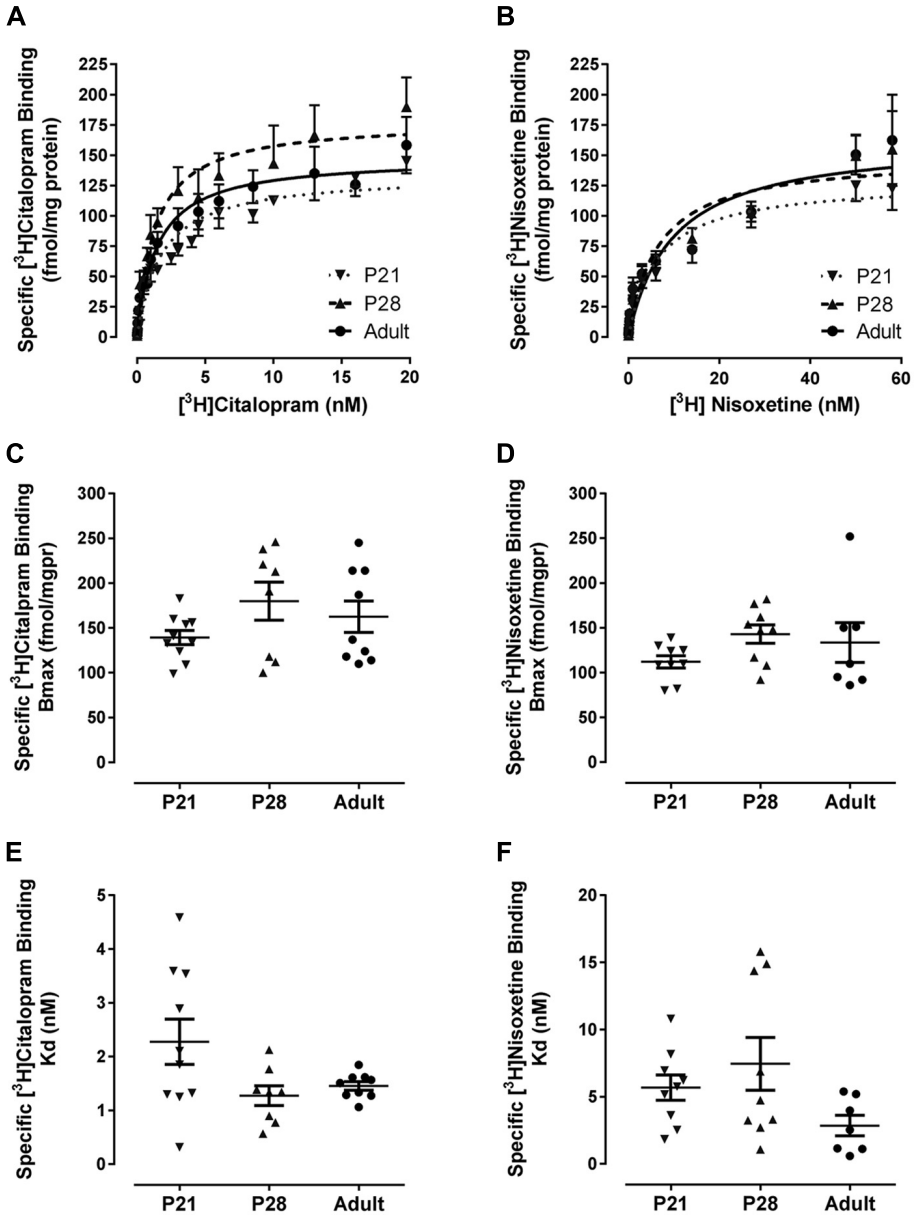

FIGURE 3 | Specific binding of [^{3}H]citalopram to SERT and [^{3}H]nisoxetine to NET in hippocampal membrane homogenates from male P21(▼) P28 (▲) and adult (●) mice. Membrane preparations were incubated with increasing concentrations of [^{3}H]citalopram or [^{3}H]nisoxetine. Non-specific binding was defined in the presence of 10 μM sertraline or 10 μM mazindol, respectively. Specific binding was obtained by subtracting non-specific binding from total binding at each ligand concentration. **(A,B)** Show saturation binding isotherms for binding of [^{3}H]citalopram or [^{3}H]nisoxetine, respectively. B_{max} and K_d values for [^{3}H]citalopram to SERT are summarized in **(C,E)**, and for [^{3}H]nisoxetine binding to NET in **(D,F)**, respectively. For [^{3}H]citalopram sample sizes were as follows: P21 $n = 10$, P28 $n = 8$, adult $n = 9$; and for [^{3}H]nisoxetine, P21 $n = 9$, P28 $n = 9$, adult $n = 7$. Data are mean and SEM.

RELATIONSHIP BETWEEN ANTIDEPRESSANT-LIKE EFFECT AND SATURATION BINDING WITH [^{3}H]CITALOPRAM AND [^{3}H]NISOXETINE IN HIPPOCAMPUS ACROSS AGE GROUPS

Data from **Figure 2** and **Table 1** are plotted in **Figure 4** and illustrate the relationship, or lack thereof, between the ability of escitalopram and DMI to produce antidepressant-like effects in the TST and the expression and affinity values for hippocampal SERT (**Figures 4A,C**) and NET (**Figures 4B,D**). TST data plotted in **Figure 4** are the immobility times for each individual escitalopram- or DMI-treated mouse, subtracted from the mean value for immobility time of the same age saline-treated mice (i.e., data from **Figure 2**). This difference provides a measure of the magnitude of antidepressant-like response that takes into account the difference in immobility times among saline-treated mice of different ages. The ability of escitalopram to produce antidepressant-like effects in the

Table 1 | Summary of B_{max} and K_d values for [^{3}H]citalopram binding to SERT and [^{3}H]nisoxetine binding to NET in male P21, P28, and adult mice.

	P21	P28	Adult
[^{3}H]Citalopram			
B_{max} (fmol/mgpr)	139 ± 8	180 ± 21	163 ± 17
K_d (nM)	2.3 ± 0.4	1.3 ± 0.2	1.5 ± 0.1
[^{3}H]Nisoxetine			
B_{max} (fmol/mgpr)	112 ± 7	143 ± 10	136 ± 23
K_d (nM)	5.7 ± 0.9	7.4 ± 2.0	2.5 ± 0.8

[^{3}H]citalopram binding n = 8–10 per group where hippocampi were from one mouse per assay. For [^{3}H]nisoxetine binding n = 7–9 per group, but where each assay was from pooled hippocampi from two mice. For each ligand, there were no significant differences in B_{max} or K_d values among ages (Kruskal–Wallis). Data are mean and SEM.

TST increased with age, but was not associated with parallel increases in either B_{max} (**Figure 4A**) or affinity of SERT for [^{3}H]citalopram (i.e., smaller K_d values; **Figure 4D**). The ability of DMI to produce antidepressant-like effects did not change significantly across age groups. Likewise, B_{max} and K_d values for [^{3}H]nisoxetine binding to NET did not vary significantly as a function of age.

DISCUSSION

The major findings of the present study are first, that the SSRI escitalopram and the NET blocker DMI produced antidepressant-like effects in mice as young as P21, the youngest age tested; second, that the magnitude of antidepressant-like response to escitalopram increased with age but was not paralleled by increasing expression or affinity of hippocampal SERT; and third that the magnitude of antidepressant-like response to the tricyclic, DMI, as well as expression and affinity of hippocampal NET, did not differ significantly among P21, P28, and adult mice. These findings support the utility of juvenile mice to study antidepressant-like activity of drugs. Moreover, our finding that juvenile mice are less sensitive to the antidepressant-like effect of escitalopram than adults, parallels clinical data reporting that, compared to adults, children have a relatively poor therapeutic response to the SSRIs, fluoxetine, and

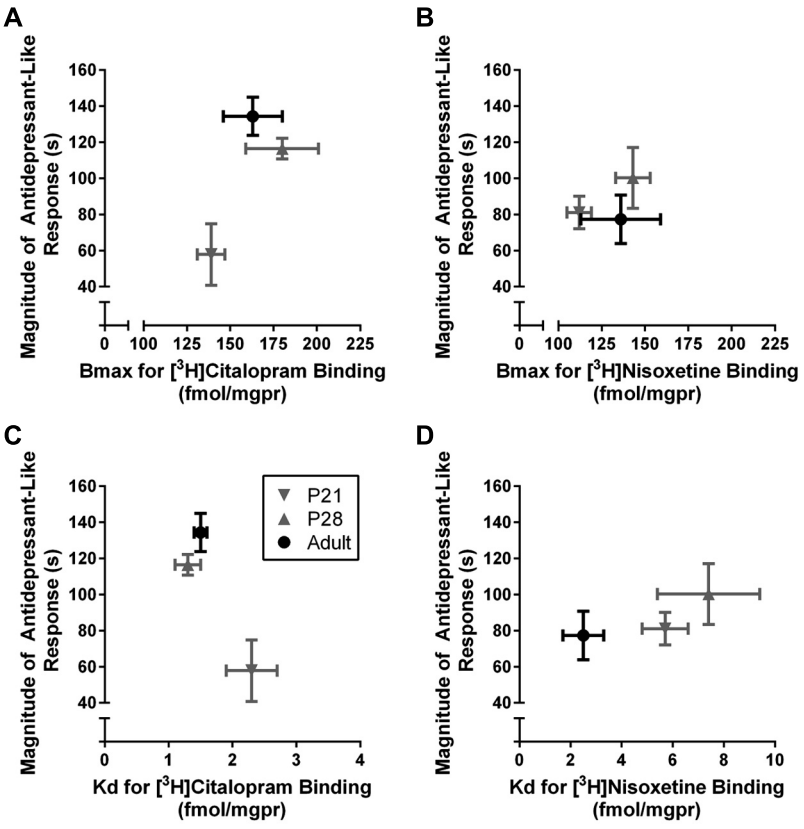

FIGURE 4 | Relationship between antidepressant-like response and hippocampal SERT and NET expression and affinity in P21, P28, and adult male mice. The magnitude of antidepressant-like response to the SERT blocker, escitalopram, increased with age but neither B_{max} **(A)** nor K_d **(C)** values for [^{3}H]citalopram binding to SERT varied significantly with age. The magnitude of antidepressant-like response to the NET blocker, DMI, as well as B_{max} **(B)** and K_d **(D)** values for [^{3}H]nisoxetine binding to NET remained relatively constant across ages. Data are re-plotted from **Figure 2** and **Table 1**. TST data from **Figure 2** are plotted on the *y*-axis as "magnitude of antidepressant-like response," defined as the immobility time for individual drug-treated mice subtracted from the mean immobility time of same aged saline-treated mice; the larger the number, the greater the antidepressant-like effect. Sample sizes are the same as reported in legends to **Figures 2,3** and **Table 1**. Data are mean and SEM.

escitalopram, the only two FDA approved antidepressants for this young population.

In rodents, postnatal days 21–27 are considered the juvenile period and postnatal days 28–42 equivalent to early adolescence (Spear, 2000; Bylund and Reed, 2007). To date only a few studies have investigated antidepressant-like activity of drugs in adolescent mice and none have studied juvenile mice. Adolescent mice (P28 or P35) were found to be sensitive to the antidepressant-like effect of both SSRIs and tricyclics in the FST (Bourin et al., 1998; David et al., 2001a,b; Mason et al., 2009). Only one study has used the TST to investigate antidepressant-like activity in adolescent mice (P35) and, as for studies using the FST, found that both SSRIs and tricyclics were effective in reducing immobility time. Consistent with these studies, we also found that both SSRI and tricyclic classes of antidepressant effectively reduced immobility time of adolescent (P28) and adult mice in the TST. Likewise, our findings in P28 and adult mice are in good agreement with reports in P28 and adult rats, where both SSRIs and tricyclic antidepressants were effective in producing antidepressant-like activity in the FST (Reed et al., 2008).

To our knowledge, this is the first report of antidepressant-like activity in P21 mice. We found that the SSRI, escitalopram, produced antidepressant-like activity in these mice; however the magnitude of this effect was less than that in adolescent (P28) or adult mice. Similarly, studies using rats aged P21 in the FST, found them to be sensitive to the antidepressant-like effects of SSRIs, including escitalopram (Reed et al., 2008). However, in this study a head-to-head comparison with P28 and adult rats was not included, making it difficult to draw conclusions as to whether the magnitude of antidepressant-like effect was less in P21 rats, compared to P28 or adult rats. Based on the present studies, where P21, P28, and adult mice were compared head-to-head, it is clear that the magnitude of escitalopram to produce antidepressant-like effects in P21 mice is less than in adult mice.

We also found that P21 mice were sensitive to the antidepressant-like effect of the tricyclic, DMI. Unlike our finding for escitalopram, the magnitude of antidepressant-like effect of DMI was similar among P21, P28, and adult mice. This finding contrasts with studies using rats, where Reed et al. (2008) found P21 rats to be insensitive to tricyclics, including DMI. Potential reasons for these differences include species (rat versus mouse), behavioral test (FST versus TST), drug dose, and route of administration. For example, the highest dose of DMI tested in studies using P21 rats was 20 mg/kg (ip; Reed et al., 2008), whereas in our studies using mice, the dose was 32 mg/kg (ip). Thus, our ability to detect antidepressant-like effects of DMI in mice as young as P21 may result in part from using a higher dose. Certainly, pharmacokinetic differences between species and across ages are also a consideration.

In the clinical setting a key difference between adult and pediatric depression is response to pharmacotherapy (Hazell et al., 1995; Kratochvil et al., 2006; Bridge et al., 2007; Bylund and Reed, 2007; Hazell and Mirzaie, 2013). The present studies using mice, as well as published reports using rats, show that like humans, juvenile mice, and rats respond differently to antidepressant drugs. While there are some apparent discrepancies in reported findings, particularly those relating to the emergence of antidepressant-like activity of DMI, there are numerous factors that may account for these; some of which have been touched on already (e.g., dose, species, test). With regard to the clinical setting, it is important to keep in mind that therapeutic benefit is also contingent upon tolerability of the drug. Thus, although tricyclics are not approved for use in children and adolescents, data from clinical trials have been mixed, with some studies reporting that tricyclics lowered depression scores in adolescents, while others found tricyclics to be therapeutically ineffective (Hazell and Mirzaie, 2013). However, consistent with the mechanism of action of tricyclic antidepressants, these drugs were more likely than placebo to produce adverse side effects, including vertigo, tremor, low blood pressure, and dry mouth. Thus, due to inconclusive demonstrations of therapeutic benefit in young humans, and the possibility of harmful side effects, or increased sensitivity to adverse side-effects in this patient population, tricyclic antidepressants are not prescribed for children and adolescents.

The key finding from the present study is that it is possible to detect antidepressant-like activity of drugs in mice as young as P21. This finding opens the door for studies geared to understanding the mechanisms underlying the relatively poor therapeutic response of young humans to SSRIs, which in turn paves the way for identifying treatments with improved therapeutic efficacy. It is worth emphasizing that essentially nothing is known about the mechanisms of antidepressant activity in juvenile and adolescent mice. Rat studies have led the way, but even then, knowledge is not extensive (for review, see Bylund and Reed, 2007) with many unknowns remaining. For example, the effect of antidepressants can be dependent on relative rates of neurotransmitter synthesis and it is not yet known if the activity of neurotransmitter synthesizing enzymes (e.g., tryptophan hydroxylase, tyrosine hydroxylase) varies during these postnatal periods in mice. Here, we began to investigate possible mechanisms underlying the divergent response of juvenile mice to SSRIs and tricyclic antidepressants by first quantifying the expression and affinity of their target proteins, SERT and NET, in hippocampus.

Hippocampus was selected for these initial studies given its importance in mood and antidepressant drug effects (Campbell and McQueen, 2004). In rats the delayed emergence of antidepressant-like activity of tricyclics is thought to be related to delayed maturation of the noradrenergic system compared with the serotonergic system (Murrin et al., 2007). As far as NET is concerned, the primary target of DMI, its expression during postnatal development in rat brain, measured using quantitative autoradiography, is age and brain region dependent. NET expression increases rapidly across brain regions between P10 and P15, and attains adult levels in some regions (e.g., cortex, CA1 and CA2 regions of hippocampus, dentate gyrus, amygdala, striatum) that are maintained into adulthood; in others regions (e.g., CA3 region of hippocampus) NET expression attains adult levels at P15, but then decreases at P20 before returning to adult levels at P25 (Sanders et al., 2005). To date there are no reports on the development of expression of NET in mouse brain over the postnatal ages studied here. Our data show that in hippocampus, NET expression was similar in P21, P28, and adult mice. Likewise, the affinity of NET for [^{3}H]nisoxetine was similar across

these ages. Data from adult mouse hippocampus presented here are in general agreement with those reported by others [e.g., B_{max} 127 ± 5 fmol/mgpr and K_d 0.7 ± 0.05 nM, C57Bl/6 male mice (Csölle et al., 2013)], and in adult rat cerebral cortex [B_{max} 97 ± 12 fmol/mgpr and K_d 0.8 ± 0.11 nM (Tejani-Butt, 1992)]. Our data are also consistent with the quantitative autoradiography measures of NET in rat hippocampus reported by Sanders et al. (2005), where, with the exception of CA3 region, NET expression did not vary across juvenile, adolescent, and adult ages. Given that we carried out saturation binding assays in homogenates taken from whole hippocampus it would be unlikely we would detect any age-dependent changes in a sub-region of hippocampus (such as CA3). At the expense of anatomical resolution, our saturation binding approach afforded a measure of transporter affinity, which to our knowledge has not been previously reported for P21 or P28 mouse or rat. Our findings in mouse hippocampus show that NET expression and affinity are at adult levels by P21. Given that the antidepressant-like effects of DMI in the TST were also similar among juvenile, adolescent, and adult mice, it appears that NET expression and affinity in hippocampus parallels DMI's antidepressant efficacy in mice. It must be recognized, however, that this does not rule out the possibility that DMI's antidepressant-like activity in the TST depends on NET expression in other regions. Further studies are needed to determine the brain region(s) and mechanisms (e.g., transporters, receptors) that mediate antidepressant-like behavioral activity in the TST following administration of NET blockers, and how this may vary with age.

Even less is known about the postnatal development of expression and affinity of SERT in mice. Quantitative autoradiography studies to date indicate that in rats, SERT expression reaches adult levels between birth and P21 (Zhou et al., 2000; Galineau et al., 2004; Bylund and Reed, 2007). Consistent with these findings in rats, we found that SERT expression in hippocampus of P21 and P28 mice was similar to that in adults. We also found that the affinity of hippocampal SERT for [^{3}H]citalopram was equivalent among P21, P28, and adult mice. To our knowledge there are only two reports of [^{3}H]citalopram binding using adult C57Bl/6 mouse hippocampal homogenate preparations. Our group previously reported values in good agreement with those reported here [B_{max} 171 ± 20 fmol/mgpr; K_d 1.1 ± 0.2 nM (Gould et al., 2011)]. Another group reported a higher B_{max} (555 ± 35 fmol/mgpr) but a similar K_d (1.2 ± 0.1 nM; Csölle et al., 2013). Of note in the present study, even though statistical analyses did not reveal significant differences in affinity of hippocampal SERT for [^{3}H]citalopram among ages, the variance of the K_d values differed significantly among ages. As is clear in **Figure 3E**, K_d values varied from 0.3 to 4.6 nM in juveniles, 0.6 to 2.1 nM in adolescents and 1.0 to 1.8 nM in adults. Thus, the spread in K_d values dropped from 4.3 nM in P21 mice, to 1.5 nM in P28 mice and to 0.8 nM in adult mice. These data suggest that juvenile and adolescent periods may be critical periods in development where, although the density of SERT is at adult expression levels, the functional activity (affinity) of SERT is undergoing a transition to that of the adult. In the case of the present data, approximately half of P21 mice had K_d values in line with those of adults, and the remainder had K_d values two or more fold greater (i.e., lower affinity for [^{3}H]citalopram). Based on these initial data, it is tempting to speculate that this variability in when the "switch" from juvenile to adult SERT affinity occurs, accounts in part for the variability in individual response to SSRIs in pediatric depression.

These studies are, to our knowledge, the first to obtain B_{max} and K_d values for [^{3}H]citalopram binding to SERT and [^{3}H]nisoxetine binding to NET, two of the most prominent targets of currently available antidepressant drugs, in juvenile and adolescent mice, and the results are in agreement with the few existing reports from adult mice (Gould et al., 2011; Csölle et al., 2013). The present findings raise the possibility that, although SERT expression may be at or near adult levels in P21 mice, the large variability in affinity state of SERT for SSRIs may account, at least in part, for the lower clinical effectiveness of SSRIs in children. Showing that the TST is a relevant behavioral assay of antidepressant-like activity in juvenile (P21) and adolescent (P28) mice, sets the stage for future studies of the mechanisms underlying the antidepressant response in these young populations.

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REFERENCES

Andreasen, J. T., and Redrobe, J. P. (2009). Antidepressant-like effects of nicotine and mecamylamine in the mouse forced swim and tail suspension tests: role of strain, test and sex. *Behav. Pharmacol.* 20, 286–295. doi: 10.1097/FBP.0b013e32832c713e

Bourin, M., Colombel, M. C., Redrobe, J. P., Nizard, J., Hascoët, M., and Baker, G. B. (1998). Evaluation of efficacies of different classes of antidepressants in the forced swimming test in mice at different ages. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 22, 343–351. doi: 10.1016/S0278-5846(98)00009-8

Bridge, J. A., Iyengar, S., Salary, C. B., Barbe, R. P., Birmaher, B., Pincus, H. A., et al. (2007). Clinical response and risk for reported suicidal ideation and suicide attempts in pediatric antidepressant treatment: a meta-analysis of randomized control trials. *JAMA* 297, 1683–1696. doi: 10.1001/jama.297.15.1683

Bujoreanu, S., Benhayon, D., and Szigethy, E. (2011). Treatment of depression in children and adolescents. *Pediatr. Ann.* 40, 548–555. doi: 10.3928/00904481-20111007-05

Bylund, D. B., and Reed, A. L. (2007). Childhood and adolescent depression: why do children and adults respond differently to antidepressant drugs? *Neurochem. Int.* 51, 246–253. doi: 10.1016/j.neuint.2007.06.025

Campbell, S., and McQueen, G. (2004). The role of the hippocampus in the pathophysiology of major depression. *J. Psychiatr. Neurosci.* 29, 417–426.

Castagne, V., Moser, P., Roux, S., and Porsolt, R. D. (2011). Rodent models of depression: forced swim and tail suspension behavioral despair tests in rats and mice. *Curr. Protoc. Neurosci.* Chapter 8, Unit 8.10A. doi: 10.1002/0471142301.ns0810as55

Csölle, C., Baranyi, M., Zsilla, G., Kittel, A., Gölöncsér, F., Illes., et al. (2013). Neurochemical changes in the mouse hippocampus underlying the antidepressant effect of genetic deletion of P2X7 receptors. *PLoS ONE* 8:e66547. doi: 10.1371/journal.pone.0066547

Cryan, J. F., and Holmes, A. (2005). The ascent of mouse: advances in modeling human depression and anxiety. *Nat. Rev. Drug Discov.* 4, 775–790. doi: 10.1038/nrd1825

Cryan, J. F., Mombereau, C., and Vassout, A. (2005). The tail suspension test a model for assessing antidepressant activity: review of pharmacological studies in mice. *Neurosci. Biobehav. Rev.* 29, 571–625. doi: 10.1016/j.neubiorev.2005.03.009

D'Amato, R. J., Largent, B. L., Snowman, A. M., and Snyder, S. H. (1987). Selective labeling of serotonin uptake sites in rat brain by [3H]citalopram contrasted to labeling of multiple sites by [3H]imipramine. *J. Pharmacol. Exp. Ther.* 242, 364–371.

David, D. J. P., Bourin, M., Hascoët, M., Colombel, M. C., Baker, G. B., and Jolliet, P. (2001a). Comparison of antidepressant activity in 4- and 40-week-old male mice in the forced swimming test: involvment of 5-HT1A and 5-HT1B receptors in old mice. *Psychopharmacology* 153, 443–449. doi: 10.1007/s002130000588

David, D. J. P., Dhonnchadha, B. A. N., Jolliet, P., Hascoët, M., and Bourin, M. (2001b). Are there gender differences in the temperature profile of mice after acute antidepressant administration and exposure to tow animal models of depression. *Behav. Brain Res.* 119, 203–211. doi: 10.1016/S0166-4328(00)00351-X

Daws, L. C., and Gould, G. G. (2011). Ontogeny and regulation of the serotonin transporter: providing insight into human disorders. *Pharmacol. Ther.* 131, 61–79. doi: 10.1016/j.pharmthera.2011.03.013

Galineau, L., Kodas, E., Guilloteau, D., Vilar, M. P., and Chalon, S. (2004). Ontogeny of the dopamine and serotonin transporters in the rat brain: an autoradiographic study. *Neurosci. Lett.* 363, 266–271. doi: 10.1016/j.neulet.2004.04.007

Gould, G. G., Hensler, J. G., Burke, T. F., Benno, R. H., Onaivi, E. S., and Daws, L. C. (2011). Density and function of central serotonin (5-HT) transporters, 5-HT1A and 5HT2A receptors, and effects of their targeting on BTBR T+tf/J mouse social behavior. *J. Neurochem.* 116, 291–303. doi: 10.1111/j.1471-4159.2010.07104.x

Hazell, P., and Mirzaie, M. (2013). Tricyclic drugs for depression in children and adolescents. *Cochrane Database Syst. Rev.* 6. doi: 10.1002/14651858.CD002317.pub2

Hazell, P., O'Connell, D., Heathcote, D., Robertson, J., and Henry, D. (1995). Efficacy of tricylcic drugs in treating child and adolescent depression: a meta-analysis. *BMJ* 310, 897–901. doi: 10.1136/bmj.310.6984.897

Hetrick, S. E., McKenzie, J. E., and Merry, S. N. (2010). The use of SSRIs in children and adolescents. *Curr. Opin. Psychiatr.* 23, 53–57. doi: 10.1097/YCO.0b013e328334bc92

Hetrick, S. E., Merry, S. N., McKenzie, J., Sindahl, P., and Proctor, M. (2009). Selective serotonin reuptake inhibitors (SSRIs) for depressive disorders in children and adolescents (Review). *Cochrane Database Syst. Rev.* 1–91.

Jones, M. D., and Lucki, I. (2005). Sex differences in the regulation of serotonergic transmission and behavior in 5-HT receptor knockout mice. *Neuropsychopharmacology* 30, 1039–1047. doi: 10.1038/sj.npp.1300664

Kessler, R. C., Avenevoli, S., and Reis Merikangas, K. (2001). Mood disorders in children and adolescents: an epidemiologic perspective. *Biol. Psychiatry* 49, 1002–1014. doi: 10.1016/S0006-3223(01)01129-5

Kratochvil, C., Emslie, G., Silva, S., McNulty, S., Walkup, J., Curry, J., et al. (2006). Acute time to response in the Treatment of Adolescents with Depression Study (TABS). *J. Am. Acad. Child Adolesc. Psychiatry* 45, 1412–1418. doi: 10.1097/01.chi.0000237710.73755.14

Mason, S. S., Baker, K. B., Davis, K. W., Pogorelov, V. M., Malbari, M. M., Ritter, R., et al. (2009). Differential sensitivity to SSRI and tricyclic antidepressants in juvenile and adult mice of three strains. *Eur. J. Pharmacol.* 602, 306–315. doi: 10.1016/j.ejphar.2008.11.010

Murrin, L. C., Sander, J. D., and Bylund, D. B. (2007). Comparison of the maturation of the adrenergic and serotonergic neurotransmitter systems in the brain: implications for differential drug effects on juveniles and adults. *Biochem. Pharmacol.* 73, 1225–1236. doi: 10.1016/j.bcp.2007.01.028

O'Leary, O. F., Bechtholt, A. J., Crowley, J. J., Hill, T. E., Page, M. E., and Lucki, I. (2007). Depletion of serotonin and catecholamines block the acute behavioral response to different classes of antidepressant drugs in the mouse tail suspension test. *Psychopharmacology* 192, 357–371. doi: 10.1007/s00213-007-0728-9

Pechnick, R. N., Bresee, C. J., Manalo, C. M., and Poland, R. E. (2008). Comparison of the effects of desmethylimipramine on behavior in the forced swim test in peripubertal and adult rats. *Behav. Pharmacol.* 19, 81–84. doi: 10.1097/FBP.0b013e3282f3d07f

Reed, A. L., Happe, H. K., Petty, F., and Bylund, D. B. (2008). Juvenile rats in the forced-swim test model the human response to antidepressant treatment for pediatric depression. *Psychopharmacology* 197, 433–441. doi: 10.1007/s00213-007-1052-0

Sanchez, C., Bergqvist, P. B. F., Brennum, L. T., Gupta, S., Hogg, S., Larsen, A., et al. (2003). Escitalopram, the S-(+)-enantiomer of citalopram, is a selective serotonin reuptake inhibitor with potent effects in animal models predictive of antidepressant and anxiolytic activities. *Psychopharmacology* 167, 353–362.

Sanders, J. D., Happe, H. K., Bylund, D. B., and Murrin, L. C. (2005). Development of the norepinephrine transporter in the rat CNS. *Neuroscience* 130, 107–117. doi: 10.1016/j.neuroscience.2004.09.014

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Behav. Rev.* 24, 417–463. doi: 10.1016/S0149-7634(00)00014-2

Steru, L., Chermat, R., Thierry, B., and Simon, P. (1985). The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology* 85, 367–370. doi: 10.1007/BF00428203

Tejani-Butt, S. (1992). [3H]nisoxetine: a radioligand for quantitation of norepinephrine uptake sites by autoradiography or homogenate binding. *J. Pharmacol. Exp. Ther.* 260, 427–436.

Tsapakis, E. M., Soldani, F., Tondo, L., and Baldessarini, R. J. (2008). Efficacy of antidepressants in juvenile depression: meta-analysis. *Br. J. Psychiatry* 193, 10–17. doi: 10.1192/bjp.bp.106.031088

Zhou, F. C., Sari, Y., and Zhang, J. K. (2000). Expression of serotonin transporter protein in developing rat brain. *Brain Res. Dev. Brain Res.* 119, 33–45. doi: 10.1016/S0165-3806(99)00152-2

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Acute dietary tryptophan manipulation differentially alters social behavior, brain serotonin and plasma corticosterone in three inbred mouse strains

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ABSTRACT

Clinical evidence indicates brain serotonin (5-HT) stores and neurotransmission may be inadequate in subpopulations of individuals with autism, and this may contribute to characteristically impaired social behaviors. Findings that depletion of the 5-HT precursor tryptophan (TRP) worsens autism symptoms support this hypothesis. Yet dietetic studies show and parents report that many children with autism consume less TRP than peers. To measure the impact of dietary TRP content on social behavior, we administered either diets devoid of TRP, with standard TRP (0.2 g%), or with 1% added TRP (1.2 g%) overnight to three mouse strains. Of these, BTBRT^{+}*Itpr3*^{tf}/J and 129S1/SvImJ consistently exhibit low preference for social interaction relative to C57BL/6. We found that TRP depletion reduced C57BL/6 and 129S social interaction preference, while TRP enhancement improved BTBR sociability ($p < 0.05$; $N = 8$–10). Subsequent marble burying did not differ among diets or strains. After behavior tests, brain TRP levels and plasma corticosterone were higher in TRP enhanced C57BL/6 and BTBR, while 5-HT levels were reduced in all strains by TRP depletion ($p < 0.05$; $N = 4$–10). Relative hyperactivity of BTBR and hypoactivity of 129S, evident in self-grooming and chamber entries during sociability tests, were uninfluenced by dietary TRP. Our findings demonstrate mouse sociability and brain 5-HT turnover are reduced by acute TRP depletion, and can be enhanced by TRP supplementation. This outcome warrants further basic and clinical studies employing biomarker combinations such as TRP metabolism and 5-HT regulated hormones to characterize conditions wherein TRP supplementation may best ameliorate sociability deficits.

1. Introduction

Sociability deficits, specifically interpersonal interaction impairments such as social anxiety, withdrawal, inattentiveness, or lack of social motivation are characteristic of autism spectrum disorders. Serotonin (5-HT) system dysfunctions are implicated in some forms of autism, and may contribute to characteristic social interaction impairments (Lam et al., 2006; Rubin et al., 2013; Yang et al., 2014). During fetal and juvenile brain development, 5-HT plays many critical roles (Daws and Gould, 2011). Clinical and basic research findings indicate that 5-HT-regulated brain developmental trajectories are disrupted in autism, either via deficient or excessive central 5-HT availability (Chandana et al., 2005; Azmitia et al., 2011; Madden and Zup, 2014; Yang et al., 2014).

Among individuals with autism, brain 5-HT availability and neurotransmission are variable, since a diverse range of genetic and environmental risk factors can manifest in common core behavioral deficits (Unwin et al., 2013; Whitehouse and Stanley, 2013). Yet subpopulations with distinct autism phenotypes can be identified, including a group with physiological markers and behavioral symptoms consistent with central hyposerotonemia (Brune et al., 2006; McNamara et al., 2008; Veenstra-VanderWeele et al., 2012). Such markers comprise reduced 5-HT transporter binding in frontal cortex (Makkonen et al., 2008; Nakamura et al., 2010), low oxytocin and low melatonin levels (Alabdali et al., 2014; Ruggeri

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et al., 2014). Selective 5-HT reuptake inhibitors (SSRIs) improve autism symptoms in some patients (West et al., 2009; Kumar et al., 2012; Hollander et al., 2012; Politte et al., 2014),[1] suggesting 5-HT neurotransmission may be reduced and/or brain 5-HT depleted.

Tryptophan (TRP) is the essential amino acid 5-HT precursor. Acute TRP depletion can be used to assess the prognosis of patients with depression to benefit from 5-HT-based treatments (Delgado, 2006; Toker et al., 2010). With TRP depletion, depression symptoms worsen and cognitive functions decline in patients that responded favorably to SSRI treatments, or in individuals with high 5-HT turnover rates (Bell et al., 2001; Delgado, 2006; Feder et al., 2011). TRP depletion in individuals with autism likewise worsens core behavior symptoms, indicating heightened sensitivity to fluctuations in TRP and 5-HT availability (McDougle et al., 1993, 1996). On the other hand, increasing dietary TRP intake ameliorated autism symptoms in a case study (Beretich, 2009). This presents a paradox, in light of reports that many individuals with autism prefer foods with relatively low TRP content or have aversions to high dietary protein content (Kidd, 2002; Arnold et al., 2003; Herndon et al., 2009; Hyman et al., 2012; Johnson et al., 2014).

Given this, we tested the hypothesis that acute dietary TRP depletion should impair social behavior, while TRP enhancement might improve it. Inbred mice expressing the high-functioning TRP hydroxylase 2 (Tph2) enzyme isoform to convert TRP to 5-HT, with well-characterized sociability phenotypes (Carneiro et al., 2009; Moy et al., 2007) were used. These included socially deficient BTBR T+ Itpr3tf/J (BTBR) and 129S1/SvImJ (129S), and relatively gregarious C57BL/6J (C57) mice. Preferences for social interaction and novelty, chamber entries, self-grooming during sociability tests and subsequent marble burying were compared among strains and overnight TRP diet treatments. After behavior tests, brain TRP, 5-HT turnover and plasma corticosterone (CORT) – since it can be suppressed by central 5-HT transmission (Gould et al., 2014) – were measured in tissues collected from all strain × diet treatment groups to assess their central 5-HT status.

2. Methods

2.1. Mice and acute dietary tryptophan manipulation

All procedures involving live mice were approved by the UTHSCSA Institutional Animal Care and Use Committee, and were in accordance with current NIH guidelines. Mice tested were fifth and sixth generation male offspring bred in the laboratory animal facilities at The University of Texas Health Science Center at San Antonio (UTHSCSA), San Antonio, TX. BTBR, C57 and 129S, founders came from Jackson Laboratory (Bar Harbor, ME, USA).

Mice were maintained at 22–25 °C on 14:10 light dark cycles, with lights on at 0700 h, and *ad libitum* access to Teklad LM-485 mouse/rat irradiated food pellets (#7912, Harlan, Madison, WI) and water in cages lined with wood-chip bedding that were changed bi-weekly. Mice were weaned at postnatal days 20–22 and were housed in same-sex groups of 3–5 per cage. Dietary TRP manipulations and behavior tests were conducted in 3–4 month-old males. For 24–30 h prior to behavior testing (beginning 0900 or 1000 h), mice had *ad libitum* access to purified ingredient or "open source" standard diets with either a) control levels of TRP (2.1 g/kg or 0.2% = green pellets), b) diet devoid of TRP (-TRP, 0% = red), or c) diet with 1% added TRP (+TRP 12.6 g/kg = yellow) from Research Diets Inc. (New Brunswick, NJ). Nutritional information for open-standard purified diets and the Teklad chow the mice were reared and maintained on are provided in Table 1.

2.2. Sociability tests, self grooming during tests and subsequent marble burying

Preference tests for social interaction and social novelty were performed in three chambered testing arenas between 9:00 and 16:00 h CST, as described in prior studies (Gould et al., 2011, 2014; Silverman et al., 2010; Yang et al., 2011). Conditioning and sociability tests were conducted under low red lighting (16 lux). A daily experiment schedule is provided, and sociability-testing arena illustrated in the online supplement (Appx A.1.a and b). Subject mice (4–6 tested in different arenas at the same time, 1 per treatment group) were brought from housing to the testing room 30 min prior to testing 24, 26 or 28 h (typically 0900, 1100 or 1400 h CST) after diets were administered to acclimate. Next, subjects acclimated to and explored the sociability arenas for 20 min. Then, subjects were confined to the center chamber for ≥1 min while pre-conditioned 'strangers' (novel male 129S mice, 8–10 weeks old) and novel objects (empty wire cages) were placed on either end chamber. Stimulus placement for testing was randomized and counter-balanced among groups. Ten min tests were videorecorded for subsequent data collection. Preference for social interaction was tested in the first 10 min with a stranger mouse in a cup cage at one end and an empty cage at the other end chamber. Then subjects were re-confined in the center while new strangers (stranger #2) were placed under empty cages and old strangers (stranger #1) were re-positioned in the arena (Appendix A.1.b). Preference for social novelty was measured in the second 10 min phase. Between subjects strangers were returned to home cages, and arenas cleaned with 70% ethanol and dried with paper towels.

Data collected by treatment-blind observers from 10 to 11 min videorecordings of social interaction and novelty preference tests included time spent in chambers, sniffing and grooming. Chamber dwelling was tracked as subjects entered new chambers by recording the time and each chamber entered and into a spreadsheet, subtracting the exit times to determine each dwelling duration, and adding durations separately for each chamber and each test phase. Sniffing was recorded by timer when a subject directed its nose toward strangers or novel objects (cup cages) from a distance of <1 cm, and ended when they turned their head or stepped away.

Table 1
Comparison of nutrient content of sustaining mouse chow that was given prior to study to the experimental open standard diet that was acutely administered before testing.

Dietary component	Teklad LM-485 (irradiated 7012, Harlan)	Exp. open standard (A11022501, research diets)
Macronutrients (% kcal)		
Protein	25	18
Carbohydrates	58	66
Fat	17	16
Fiber (g%)	14%	10%
Essential[a] L-Amino Acids (g%)		
Arginine	1.2	0.6
Histidine	0.5	0.4
Isoleucine	0.8	0.7
Leucine	1.7	1.5
Lysine	1.0	1.3
Methionine	0.4	0.5
Phenylalanine	0.9	0.8
Threonine	0.8	0.7
Tryptophan	0.3	0.2 (−TRP 0, +TRP 1.2)
Valine	0.9	0.9
Vitamins (IU/g)		
A	30	4000
D	2.4	1000
E	0.2	50
Vitamins (mg/kg)		
Menadione (K3)	80	0.5
B-complex		
Thiamine (B1)	95	6
Riboflavin (B2)	14	6
Niacin (B3)	100	30
Pantothenate (B5)	87	16
Pyroxidine (B6)	17	7
Biotin (B7)	0.8	0.2
Folate (B9)	7	2
Cobaltamin (B12)	0.09	0.01
Minerals (g%)		
Calcium	1.0	0.9
Phosphorus	0.7	0.3
Sodium	0.3	1.7
Potassium	0.8	0.6
Chloride	0.5	1.0
Magnesium	0.2	0.5
Minerals (mg/kg)		
Zinc	63.0	29.0
Manganese	93.0	59.0
Copper	23.0	6.0
Iodine	3.0	0.2
Iron	240.0	37.0

[a] For juvenile mice, per John and Bell, 1976.

[1] However, parallel benefits of SSRI treatment are frequently muted or absent in children with autism (Henry et al., 2009; Williams et al., 2013; Politte et al., 2014).

Self-grooming was recorded by timer when a stationary subject was observed to lick or use forepaws to smooth its head, body or tail and ended when they stopped moving its head and paws or stepped away from the site where grooming occurred.

2.3. Marble burying

Immediately following each bought of social novelty testing, each subject was transferred to a 50 × 28 × 23 cm cage filled to a depth of 8 cm with bedding, on top of which was placed 15 blue flattened marbles spaced evenly apart in a 3 × 5 grid. Cages were covered with filter tops and mice had 30 min to bury marbles. For each mouse marbles ≥2/3 buried were tallied.

2.4. Whole brain TRP levels and 5-HT turnover

After marble burying (at 1100, 1300 or 1600 h CST) subject mice were sacrificed by decapitation, brains were harvested and frozen at −80 °C, and trunk blood collected into tubes containing 25 μl of 20 mM ethylenediaminetetraacetic acid (Sigma, St Louis, MO).

Levels of TRP, 5-HT and its metabolite 5-hyroxyindoleacetic acid (5-HIAA) were measured in whole brain tissue by high performance liquid chromatography (HPLC) with electrochemical (coulometric) detection. HPLC was performed as in prior studies (Callaghan et al., 2007), with minor modifications (e.g. software and system updates). In summary, brain samples were homogenized in methanol (1:9 w/v) followed by centrifugation to remove protein. Aliquots of the supernatants were dried to residue, re-dissolved in mobile phase, and then injected into the HPLC system using an isocratic mobile phase and a Alltima C18 analytical column. Peak areas of analytes were compared against a linear regression of calibrators using Empower software (Waters Corp). Final concentrations were expressed as pg/mg brain wet weight.

2.5. Plasma corticosterone (CORT) levels

Plasma isolated by centrifugation (≈3000 rpm) for 10 min at 4 °C was frozen at −80 °C until use. CORT levels were measured following the 'small sample protocol' for ELISA (ADI-900-097, Enzo Life Sciences, Farmingdale, NY) on a plate reader (Molecular Devices, Sunnyvale, CA). A non-linear standard curve was generated to determine concentrations using Prism (GraphPad, San Diego, CA).

2.6. Statistical analyses

Three-way (strain × test-phase × diet) repeated-measures multivariate analysis of variance (RM-MANOVA) comparisons of chamber-dwelling and sniffing data were performed to reveal any differences in preference between strains and among diets across social-interaction and social-novelty test-phases. Repeated measures results across strains are in Appendix (A.2 on-line supplement). Next, by strain, effects of test-phase (1. social interaction or 2. social novelty) × diet on chamber-dwelling and sniffing preferences were compared by two-way RM-ANOVA. Within each strain, diet and test-phase, significant chamber preference differences were resolved via two-tailed *t*-tests. Then, within each strain and test-phase, to compare effects of diet on each preference-related time variable, ANOVA was performed and significant differences were resolved by Fisher's least significant difference (LSD) test. Other variables such as chamber entry, self-grooming, marble burying, brain TRP, 5-HT, 5-HIAA and plasma corticosterone was compared by two-way MANOVA or ANOVA, correlation and Fisher's LSD post-hoc tests. Analyses were performed using Statistica (Statsoft, Tulsa, OK).

3. Results

3.1. Effects of dietary tryptophan manipulation on C57 sociability

During social-interaction preference tests (phase 1), diet and chamber-dwelling interacted ($F_{2,24} = 5.93$, $p < 0.01$) for C57 mice since only controls dwelled more with strangers than objects ($F_{2,24} = 3.68$, $p < 0.05$, LSD $p < 0.05$, $t_8 = 5.45$, $p < 0.001$, Fig. 1a). By contrast, +TRP and -TRP C57 dwelled in novel-object chambers moreso than controls ($F_{2,24} = 11.2$, $p < 0.001$, LSD $p < 0.001$, Fig. 1a). For C57 sniffing, diet × chamber-preference interacted ($F_{2,24} = 5.9$, $p < 0.01$) since controls sniffed strangers more than objects ($F_{2,24} = 9.5$, $p < 0.01$, $t_8 = 4.94$ $p < 0.01$, Fig. 1b), while +TRP or −TRP C57 mice sniffed novel objects more than controls ($F_{2,24} = 6.33$, $p < 0.01$, LSD $p < 0.001$, Fig. 1b).

In social-novelty preference tests (phase 2), C57 chamber preference differed among diets ($F_{1,24} = 10.71$, $p < 0.005$), as only -TRP C57 mice dwelled near stranger 2 moreso than stranger 1 ($t_8 = 2.47$, $p < 0.05$, Fig. 1c). However both −TRP and +TRP C57 mice sniffed

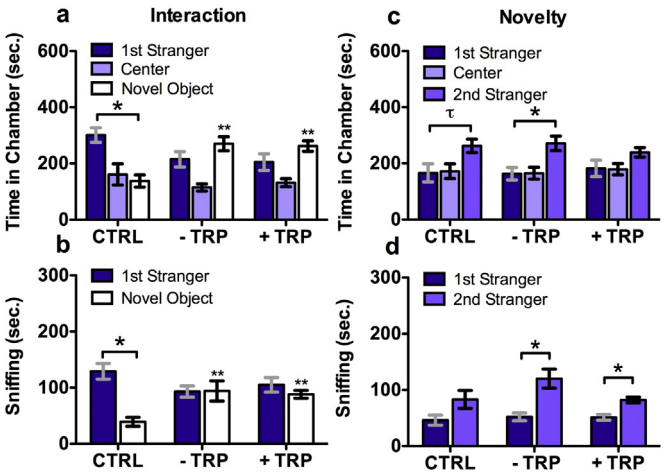

Fig. 1. C57 mouse preference for social interaction or novelty is differentially altered by acute dietary tryptophan (TRP) manipulation. Bars represent means and lines show standard error. The symbol * indicates significant preference for interaction or novelty ($p < 0.05$), τ indicates trend ($p < 0.1$) toward novelty preference, and ** indicates a change (an increase) in attention toward novel objects ($p < 0.05$). TRP enhancement (+) or depletion (−) produced a loss of interaction preference in (a) chamber dwelling and (b) sniff time. However, preference for novelty in (c) chamber dwelling or (d) sniffing was enhanced by −TRP. $N = 9$ mice/group.

stranger 2 more than stranger 1 ($F_{1,24} = 20.96$, $p < 0.001$, $t_8 = 3.63$ or 4.66, $p < 0.05$, Fig. 1d).

3.2. Effects of dietary tryptophan manipulation on 129S sociability

During social-interaction tests, diet × chambers interacted ($F_{2,23} = 5.6$, $p < 0.01$) for 129S mice. 129S controls were sociable ($F_{2,23} = 3.9$, $p < 0.05$, $t_9 = 2.42$, $p < 0.05$), while 129S on −TRP and +TRP diets had no preference for social-interaction by chamber-dwelling ($F_{2,23} = 11.95$, $p < 0.001$, LSD $p < 0.05$, Fig. 2a). −TRP 129S spent more time in the arena center than controls ($F_{2,23} = 3.9$, $p < 0.05$). For 129S sniffing, diet × chambers interacted ($F_{2,23} = 4.7$, $p < 0.05$), since controls sniffed strangers more than

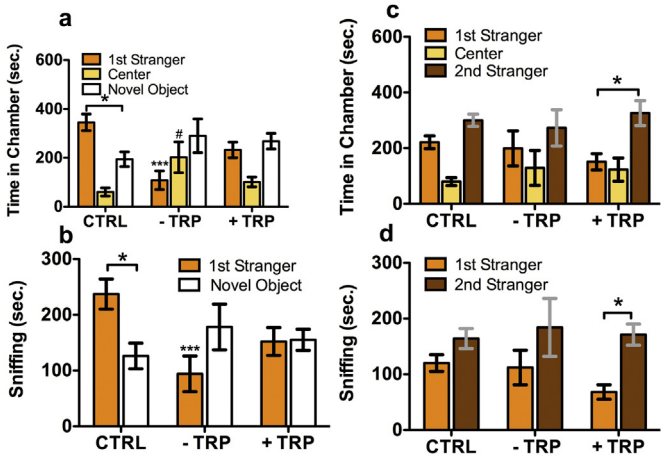

Fig. 2. 129S mouse preference for social interaction or novelty is differentially altered by dietary TRP manipulation. Graph legend is as for Fig. 1, plus # indicates significantly more time spent in the center arena ($p < 0.05$) and *** indicates significantly less time with stranger ($p < 0.05$). TRP depletion resulted in loss of social interaction preference for (a) chamber dwelling and (b) sniff time. TRP enhancement promoted preference for social novelty in (c) chamber dwelling and (d) sniff time. $N = 8–10$ mice/group.

novel-objects ($t_9 = 2.63$ $p < 0.05$), and −TRP sniffed strangers less than controls ($F_{2,23} = 6.9$, $p < 0.01$; LSD $p < 0.01$, Fig. 2b).

In 129S social-novelty preference tests +TRP mice exhibited greater preference for the second novel stranger mouse (stranger 2) than stranger 1, both in chamber-dwelling ($F_{1,23} = 6.49$, $p < 0.01$, $t_7 = 2.75$, $p < 0.05$, Fig. 2c) and sniffing ($F_{1,23} = 7.83$, $p < 0.01$, $t_7 = 3.98$, $p < 0.01$, Fig. 2d), while 129S controls displayed no such preference for novelty.

3.3. Effects of dietary tryptophan manipulation on BTBR sociability

In social-interaction tests, BTBR mice given +TRP diet exhibited enhanced sociability in chamber-dwelling ($F_{2,26} = 6.2$, $p < 0.01$, $t_8 = 2.37$, $p < 0.05$, Fig. 3a), and sniffing ($F_{2,26} = 5.4$, $p < 0.01$, $t_8 = 2.31$, $p < 0.05$, Fig. 3b) while other groups did not. BTBR given +TRP spent less time in chambers with ($F_{2,26} = 5.44$, $p < 0.05$; LSD $p < 0.05$) or sniffing ($F_{2,26} = 4.03$, $p < 0.05$; LSD $p < 0.05$) novel objects.

BTBR mice failed to exhibit any preference for the second new stranger introduced in the social-novelty phase, as measured by chamber dwelling ($F_{2,26} = 1.0$, $p = 0.3$, Fig. 3c) or social sniffing ($F_{2,26} = 3.2$, $p = 0.08$, Fig. 3d). Also there was no difference among BTBR diets in lack of social-novelty preference (chambers $F_{2,26} = 1.4$, $p = 0.26$; sniff $F_{2,26} = 0.6$, $p = 0.54$).

3.4. Chamber entries and self grooming during sociability tests

The number of chamber entries differed among strains in both social-interaction ($F_{2,73} = 26.38$, $p < 0.0001$) and social-novelty ($F_{2,73} = 13.82$, $p < 0.0001$) preference tests. 129S mice made fewer entries than C57 or BTBR mice during social interaction tests (LSD $p < 0.01$), while BTBR made more chamber entries than C57 or 129S mice (LSD $p < 0.0001$, Fig. 4a) during both test phases. BTBR on +TRP diet made more entries (66 ± 5) than BTBR controls (53 ± 8, $F_{2,73} = 3.49$, $p < 0.05$ LSD $p < 0.05$). There were no other effects or interactions of diet with strain for chamber entries during sociability testing.

Diet had no effect on self-grooming during sociability tests ($F_{2,73} = 1.61$, $p = 0.2$), but strain ($F_{2,73} = 5.20$, $p < 0.01$), test phase ($F_{2,73} = 17.96$, $p < 0.0001$) and their interaction ($F_{4,73} = 2.63$, $p < 0.05$) were significant. During social-interaction preference tests, 129S did less self grooming than BTBR or C57 mice ($F_{2,73} = 5.8$, $p < 0.01$, LSD $p < 0.005$, Fig. 4b). In social-novelty preference tests, BTBR self-groomed more than the other strains ($F_{2,73} = 3.9$, $p < 0.05$, Fig. 4b).

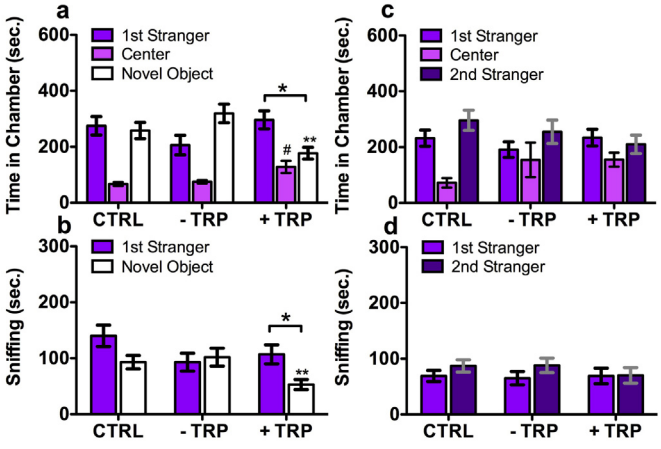

Fig. 3. BTBR mouse preference for social interaction or novelty is differentially altered by dietary TRP manipulation. Graph legend is as for Fig. 2. TRP enhancement increased preference for social interaction in (a) chamber dwelling time and (b) social sniffing, through a reduction in the attention paid to novel objects (**). There were no significant differences in the lack of preference for social novelty displayed by all treatment groups for (c) chamber dwelling or (d) sniffing time. $N = 8$–12 mice/group.

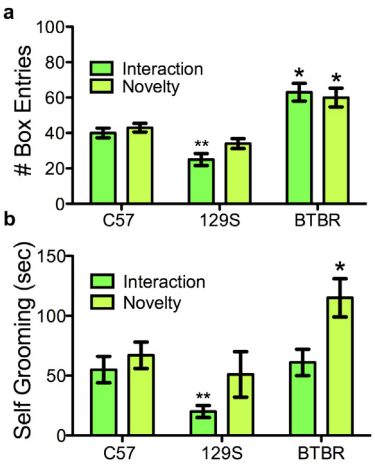

Fig. 4. Chamber entry and grooming behaviors during sociability tests were enhanced in BTBR and reduced in 129S mice. Bars represent means and lines show standard error. The symbol * indicates greater than and ** indicates less than other strains ($p < 0.05$). (a) BTBR mice made more chamber entries and 129S made fewer during sociability tests. (b) 129S did less self-grooming during preference for interaction tests than the other strains, while BTBR did more self-grooming during preference for novelty tests. $N = 8$–12 mice/group.

3.5. Marble burying after sociability testing

Diet treatment ($F_{2,73} = 1.49$, $p = 0.23$) and strain ($F_{2,73} = 1.43$, $p = 0.25$) had no significant effects on marble burying, and they did not interact ($F_{4,73} = 0.1$, $p < 0.98$). Mice in all groups buried to a similar extent on these diets, and their pooled mean was 9 ± 0.3 marbles buried.

3.6. Whole brain TRP levels and 5-HT turnover following behavior tests

Two-way MANOVA revealed significant interactions between strain and diet (Wilks' $\lambda_{16,70} = 0.18$, $p < 0.001$), with respect to whole brain wet tissue content of TRP ($F_{4,26} = 3.6$, $p < 0.05$), 5-HT ($F_{4,26} = 4.2$, $p < 0.01$), 5-HIAA ($F_{4,26} = 4.3$, $p < 0.01$) and 5-HT turnover (% 5-HIAA/5-HT $F_{4,26} = 3.2$, $p < 0.05$) in HPLC measurements of whole brains collected after behavior tests. C57 and BTBR mice had increased brain TRP content with either TRP enhancement or depletion relative to controls (Fig. 5a, LSD $p < 0.05$). As shown in Fig. 5b and c, acute TRP depletion significantly reduced (LSD $p < 0.05$) brain 5-HT and 5-HIAA content in all strains relative to controls. However TRP depletion in only C57 reduced 5-HT turnover, while TRP enhancement only in BTBR mice enhanced 5-HT turnover (Fig. 5d).

3.7. Plasma corticosterone levels following behavior tests

There was a significant interaction between strain and diet treatment ($F_{4,49} = 3.3$, $p < 0.05$), in plasma CORT levels measured in representative samples following our behavior test battery, as shown in Fig. 6. Specifically both TRP depleted and TRP enhanced C57 mice had higher CORT than those on standard control diet (LSD $p < 0.05$). Only TRP-enhanced BTBR mice had higher CORT levels than controls. By contrast, TRP-enhanced 129S had lower CORT than controls (LSD $p < 0.05$). For reference, baseline plasma CORT levels from aged-matched naïve mice were 20 ± 4 in C57, 32 ± 8 in 129S, and 28 ± 4 in BTBR ($N = 3$–5).

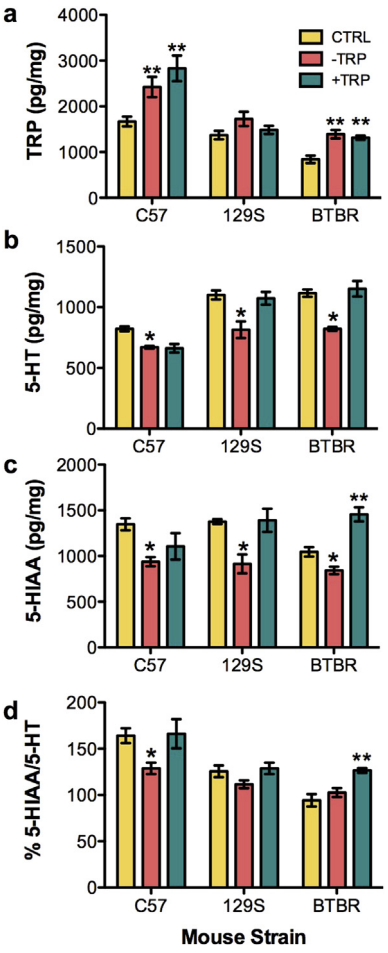

Fig. 5. Whole brain TRP and 5-HT turnover after social interaction and marble burying test battery. (a) Dietary TRP enhancement (+TRP) or depletion (−TRP) significantly increased brain TRP levels in C57 and BTBR mice (**$p < 0.05$). Depletion of TRP reduced brain (b) 5-HT levels and (c) 5-HIAA levels after behavior tests (*$p < 0.05$) in all strains, but it reduced (d) 5-HT turnover in C57 mice only. On the other hand TRP enhancement only increased 5-HT turnover in BTBR mice (d, **$p < 0.05$). $N = 4$ mice/group (randomly selected).

4. Discussion

4.1. Summary of key experimental findings

This study utilized 24–30 h of *ad libitum* feeding on purified diets to assess the impact of acute TRP manipulation on the sociability of three inbred mouse strains. Indeed, dietary TRP depletion altered murine sociability in three-chamber tests, but in different ways dependent upon mouse strain. For example in typically sociable C57 mice, preference for social-interaction was lost in −TRP mice, as they investigated novel objects more than controls (Fig. 1). Likewise, 129S on −TRP diet dwelled less with strangers, spending more time in central and novel-object chambers (Fig. 2). However in BTBR mice that usually exhibit sociability deficits, −TRP diet had little effect on social-interaction preference, while +TRP improved BTBR sociability (Fig. 3). Specifically +TRP BTBR paid less attention to novel objects, both in chamber-dwelling and sniff, and spent more time in the center (closer to strangers) relative to control or −TRP mice (Fig. 3). These findings support our hypothesis that deficits in social interaction preference can stem from reduced 5-HT availability, and parallel observations in patients with autism (McDougle et al., 1993, 1996; Beretich, 2009; Daly et al., 2012).

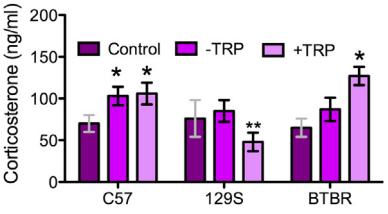

Fig. 6. Plasma corticosterone after behavior tests varied with strain and dietary TRP. The symbol * indicates greater than and ** indicates less than other strains ($p < 0.05$). In C57 mice either supplementing (+TRP) or depleting (−TRP) resulted in higher CORT relative to controls. +TRP reduced CORT significantly in 129S while it increased CORT in BTBR mice. Serum was collected after 70 min of behavior tests. $N = 4–9$ mice/group (randomly selected).

An unanticipated finding was that 129S mice on control diet were sociable. Previously, naïve 129S fed Teklad chow exhibited impaired sociability, in agreement with Moy et al. (2007), even as collinear (129S) strangers were used (Gould et al., 2014). However, distinct oxytocin-mediated responses to same vs. different mouse strains were found in other studies (Macbeth et al., 2009; Hattori et al., 2014). To assess whether collinear (129S) strangers influenced 129S sociability herein, we replicated overnight (24 h) control-diet treatment and tested 129S sociability using C57 strangers instead (Appendix A.3, Fig. 7). Even with C57 strangers, 129S controls displayed preference for social-interaction. This enhanced 129S sociability may instead stem from some aspect of the control diet, such as its composition or novelty. Likewise, purified diets may have precipitated the loss of BTBR's characteristic social-novelty preference (Fig. 3), with little effect on C57 controls (Fig. 1).

The texture of the open standard diet was similar to Teklad chow (Table 1); it has similar fat content and was not oily, and like cereal chow it was compressed to prevent crumbling. However, open standard diets are made from purified ingredients, so due to their refinement they may have a different aroma than Teklad chow (Ricci and Ulman, 2005). Hence introducing purified diets might have presented odor and flavor novelty. Yet mice in all groups consumed their diets, and feces color matched the group's diet color. While not empirically quantified, feeding did not appear to be suppressed, as comparable volume reductions to overnight feeding on Teklad diets were evident across groups.

However aside from variable TRP, other differences in macronutrient content between Teklad chow and open-standard diets were notable, and may have enhanced social interaction preference in 129S controls or reduced social novelty preference in BTBR. Specifically, vitamin A, D, and E contents were greater in purified diets. Vitamin A deficiency has anxiogenic effects (Bonhomme et al., 2014), and E deficiency alters CORT release in rats (Terada et al., 2011). Vitamin D influences sniffing and following (Kalueff et al., 2006), and can modulate 5-HT synthesis (Patrick and Ames, 2014). The level of menadione, an oxidative stressor (Giustarini et al., 2006) was reduced in purified diets. Finally, consistent with National Research Council (1995) recommended 35 mg/kg, open standard diets had 37 mg/kg of iron, while Teklad chow contained 240 mg/kg iron.

Also 129S mice on +TRP diet exhibited social-novelty preference (Fig. 2c). We postulate this change may stem from confluence of the following factors: 1) novelty of diet, 2) +TRP in diet, 3) sociability test duration and phase (social novelty test after 30 min in arena), and 4) previously characterized contextual fear-extinction deficiencies in 129S that resemble post-traumatic stress disorder (Camp et al., 2012; Temme et al., 2014). Previously we found evidence for involvement of hippocampal 5-HT_{1A} receptors in the

abnormal CORT responses of 129S mice, highlighting an important role for 5-HT in this process (Gould et al., 2014). In accord, the CORT response in +TRP 129S after behavior tests was relatively blunted (** in Fig. 6). Otherwise, plasma CORT following behavior tests differed among strain such that in C57 higher CORT in +TRP and −TRP corresponded with loss of preference for interaction, whereas higher CORT in +TRP BTBR corresponded with reduced interest in novel objects. Overall correlations between % 5-HT turnover (Fig. 5d) and plasma CORT were $r = -0.36$ ($p = 0.27$) for C57, $r = -0.59$ ($p < 0.05$) for 129S, and $r = 0.66$ ($p < 0.1$) for BTBR, more detailed correlations are presented in Appendix A4.

As with +TRP, BTBR sociability is also enhanced by treatment with the SSRI fluoxetine, indicating that deficient 5-HT neurotransmission may underlie its impaired sociability (Gould et al., 2011). Given this, it is interesting that CORT blocks ancillary transporters of 5-HT (i.e. "uptake 2") such as organic cation transporters (OCTs) from clearing extracellular 5-HT in the brain (Baganz et al., 2010; Hill et al., 2011). This lends support to the hypothesis that their blockade may be a useful strategy for treating sociability deficits, warranting further studies in this area.

4.2. Prior findings with dietary tryptophan depletion in mice

In C57 mice van Donkelaar et al. (2010) found that TRP depletion achieved by oral gavage of solutions with a high ratio of large neutral amino acids relative to TRP failed to alter serum TRP, central 5-HT levels, 5-HT metabolism or behavior in forced swim and zero mazes. Blood samples were taken 30 min prior to start, at gavage (t0) and 30, 60 and 120 min after, and brain samples were collected at 20, 40, 60 and 240 min after. The authors attributed this difference to slower TRP metabolism and higher baseline TRP in C57 mice. Rat responses to TRP manipulation also vary by strain, both when TRP-to-5-HT metabolism in the brain and behavior are considered (Jans et al., 2010). Biskup et al. (2012) compared C57 and BALB/c responses to a TRP-free nutrient solution with low methionine (Moja-De) on a schedule including post-gavage tissue measures from 90 to 330 min, and saw depression-like behaviors were augmented by TRP depletion (Biskup et al., 2012). They also discovered both strains had less TRP and reduced 5-HT and 5-HIAA levels in brain (similar to Fig. 5 herein). However, when given a balanced amino acid mixture including more TRP (0.7 g/10 kg) 5-HT synthesis was not enhanced (Biskup et al., 2012). Acute TRP depletions in mice by oral gavage are expeditious, but associated procedures such as food deprivation, restraint, forced-feeding, and repeated blood sampling, are stressful (van Donkelaar et al., 2010, 2011). Since stress-sensitive social behavior tests were our main interest, we opted instead to deplete TRP via *ad libitum* administration of purified diet overnight to achieve this end.

Modified pellet diets were employed in prior studies to examine the effects of chronic TRP depletion on rodent behaviors. In one study, dietary TRP depletion for 7 and 14 days increased immobility in forced swim, decreased TRP and 5-HT in brain, and increased serum CORT at both times in rats (Franklin et al., 2012). One-month dietary TRP depletion in C57 mice enhanced aggression, dominance and hyperactivity (Uchida et al., 2005). In C57 and BALB/c mice, Browne et al. (2012) examined chronic effects of dietary TRP manipulation (depleted, deficient, control and enhanced) in emotionality tests, and on TRP and 5-HT turnover. Pertinent to our finding of enhanced sociability in BTBR by +TRP diet (Fig. 3) was their discovery that TRP enhancement promoted nesting behavior (Browne et al., 2012).

With TRP depletion, plasma and brain TRP and 5-HT turnover was reduced and nesting and marble-burying were suppressed (Browne et al., 2012). Marble burying is typically sensitive to serotonergic manipulations (Deacon, 2006). A limitation inherent in the present study is that marble-burying was measured after sociability tests, and associated CORT increases may have obscured effects of TRP manipulation upon this measure. Yet we measured marble burying after sociability tests before and found drug (SSRI)-induced changes (Gould et al., 2011). This indicates that dietary TRP effects may be less robust than SSRI effects.

4.3. Other considerations: Tph function and TRP supplementation

Our observations that 5-HT availability can impact BTBR social behavior are paralleled in other inbred (BALB/c) and transgenic (Tph2 knock-out) mice with impaired sociability (Flood et al., 2012; Kane et al., 2012). Indeed, metabolic TRP abnormalities due to enzymatic deficiencies may contribute to brain hyposerotonemia in some patients with autism (Boccuto et al., 2013; Schwartz, 2014). In case-control studies, children with autism had elevated levels of plasma-free TRP and blood 5-HT levels, suggestive of compromised Tph2 function in brain (Hoshino et al., 1984, 1986; Coon et al., 2005). Thus, either low dietary TRP intake or compromised TRP to 5-HT conversion in brain can impair social behavior. In the latter, TRP supplements may be more problematic than helpful. However if capacity to produce 5-HT is compromised, drug interventions prolonging presence of 5-HT in the synapse, as might be achieved by OCT3 blockade (Baganz et al., 2008; Horton et al., 2013), may be of great therapeutic benefit for the treatment of sociability impairments.

Developmental depletion of cortical 5-HT by 5,7-dihydroxytryptamine in mice had transient effects on 5-HT transporter and receptor expression, yet it produced persistent increases in anxiety in response to change (Hohmann et al., 2007). On the other hand monoamine oxygenase-A knock-out mice have reduced capacity to degrade 5-HT and are aggressive, antisocial and bury many marbles, effects that were blocked by the Tph blocker p-chloro-phenylalanine ((PCPA) Bortolato et al., 2013). PCPA treatment was found to impair object recognition in mice (Alkam et al., 2011). Given this it will be of great interest learn of the effects of PCPA treatment on sociability in these strains in future studies.

4.4. Clinical relevance: dietary TRP in autism and social behavior

'Picky eating' is commonly reported among children with autism, and manifests in a limited range of acceptable foods, high frequency intake of a single food type, or food refusal (Marí-Bauset et al., 2014). Selective avoidance of proteins rich in amino acids such as TRP may relate to the fact that about one-third of patients with autism exhibit "platelet hyperserotonemia" or higher blood 5-HT levels than normal (Anderson et al., 1990; Croonenberghs et al., 2005). This biomarker may result from excess intestinal 5-HT synthesis and release, or reduced hepatic 5-HT removal (Janušonis, 2008; Gabriele et al., 2014). So TRP avoidance by some children with autism might be a response to discomfort associated with elevated gut 5-HT (Aitken, 2008; Nakamura and Hasegawa, 2009).

To the limited extent that plasma amino acids and dietary intake in children with autism have been studied, evidence of protein and nutrient malnutrition has emerged, including relatively low levels of TRP, particularly in 4 and 8 year olds; and this is even more profound with casein/gluten restricted diets (Arnold et al., 2003; Herndon et al., 2009; Kałuzna-Czaplinska et al., 2010; Hyman et al., 2012; Tanoue et al., 2012; Naushad et al., 2013). While popular among alternative-approaches to manage autism symptoms, in general the effects of restricted diets have not been well-characterized (Marcason, 2009). However there is evidence that restrictions can worsen symptoms or hinder social development in children with central hyposerotonemia and autism (Christison and

Ivany, 2006; Hjiej et al., 2008; Johnson et al., 2014). On the other hand, while TRP supplementation may ameliorate symptoms in some patients (Lakhan and Vieira, 2008), evidence for its effects in a broader population of autism patients is lacking. Hence studies on developmental and long-term effects of manipulating TRP intake are also warranted.

4.5. Conclusions: significance of experimental findings

In conclusion, we have shown that overnight *ad libitum* dietary TRP manipulation alters social behaviors in BTBR, C57 and 129S mice in different ways: C57 and 129S mice behaviors were sensitive to TRP depletion (reduced preference for social interaction) that corresponded with clinical responses in autistic patients (McDougle et al., 1993, 1996). Also enhanced social interaction preference in BTBR after TRP supplementation resembled the response of a similarly-treated autism patient (Beretich, 2009). More clinical studies examining effects of dietary TRP supplementation are needed to determine how prevalent such beneficial responses might be in the greater population of patients with autism. However, it appears that screening 5-HT regulated hormones such as cortisol, oxytocin or prolactin, could provide some insight into the functional status of 5-HT system in patients. Such screens may help to determine if TRP supplements or 5-HT-based therapeutic interventions would be beneficial on an individualized basis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2014.10.024.

References

Aitken, K.J., 2008. Intersubjectivity, affective neuroscience, and the neurobiology of autistic spectrum disorders: a systematic review. Keio J. Med. 57, 15–36.
Alabdali, A., Al-Ayadhi, L., El-Ansary, A., 2014. Association of social and cognitive impairment and biomarkers in autism spectrum disorders. J. Neuroinflammation 11, 4.
Alkam, T., Hiramatsu, M., Mamiya, T., Aoyama, Y., Nitta, A., Yamada, K., Kim, H.C., Nabeshima, T., 2011. Evaluation of object-based attention in mice. Behav. Brain Res. 220, 185–193.
Anderson, G.M., Horne, W.C., Chatterjee, D., Cohen, D.J., 1990. The hyperserotonemia of autism. Ann. N. Y. Acad. Sci. 600, 331–340.
Arnold, G.L., Hyman, S.L., Mooney, R.A., Kirby, R.S., 2003. Plasma amino acids profiles in children with autism: potential risk of nutritional deficiencies. J. Autism Dev. Disord. 33, 449–454.
Azmitia, E.C., Singh, J.S., Whitaker-Azmitia, P.M., 2011. Increased serotonin axons (immunoreactive to 5-HT transporter) in postmortem brains from young autism donors. Neuropharmacology 60, 1347–1354.
Baganz, N.L., Horton, R.E., Calderon, A.S., Owens, W.A., Munn, J.L., Watts, L.T., Koldzic-Zivanovic, N., Jeske, N.A., Koek, W., Toney, G.M., Daws, L.C., 2008. Organic cation transporter 3: keeping the brake on extracellular serotonin in serotonin-transporter-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 105, 18976–18981.
Baganz, N., Horton, R., Martin, K., Holmes, A., Daws, L.C., 2010. Repeated swim impairs serotonin clearance via a corticosterone-sensitive mechanism: organic cation transporter 3, the smoking gun. J. Neurosci. 30, 15185–15195.
Bell, C., Abrams, J., Nutt, D., 2001. Tryptophan depletion and its implications for psychiatry. Br. J. Psychiatry 178, 399–405.
Beretich, G.R., 2009. Reversal of autistic symptoms by removal of low-relative tryptophan foods: case report. Med. Hypotheses 73, 856–857.
Biskup, C.S., Sánchez, C.L., Arrant, A., Van Swearingen, A.E., Kuhn, C., Zepf, F.D., 2012. Effects of acute tryptophan depletion on brain serotonin function and concentrations of dopamine and norepinephrine in C57BL/6J and BALB/cJ mice. PLoS One 7, e35916.
Boccuto, L., Chen, C.F., Pittman, A.R., Skinner, C.D., McCartney, H.J., Jones, K., Bochner, B.R., Stevenson, R.E., Schwartz, C.E., 2013. Decreased tryptophan metabolism in patients with autism spectrum disorders. Mol. Autism 4, 16.
Bonhomme, D., Minni, A.M., Alfos, S., Roux, P., Richard, E., Higueret, P., Moisan, M.P., Pallet, V., Touyarot, K., 2014. Vitamin A status regulates glucocorticoid availability in Wistar rats: consequences on cognitive functions and hippocampal neurogenesis? Front. Behav. Neurosci. 8, 20.
Bortolato, M., Godar, S.C., Tambaro, S., Li, F.G., Devoto, P., Coba, M.P., Chen, K., Shih, J.C., 2013. Early postnatal inhibition of serotonin synthesis results in long-term reductions of perseverative behaviors, but not aggression, in MAO A-deficient mice. Neuropharmacology 75, 223–232.
Browne, C.A., Clarke, G., Dinan, T.G., Cryan, J.F., 2012. An effective dietary method for chronic tryptophan depletion in two mouse strains illuminates a role for 5-HT in nesting behaviour. Neuropharmacology 62, 1903–1915.
Brune, C.W., Kim, S.J., Salt, J., Leventhal, B.L., Lord, C., Cook Jr., E.H., 2006. 5-HTTLPR Genotype-specific phenotype in children and adolescents with autism. Am. J. Psychiatry 163, 2148–2156.
Callaghan, P.D., Owens, W.A., Javors, M.A., Sanchez, T.A., Jones, D.J., Irvine, R.J., Daws, L.C., 2007. In vivo analysis of serotonin clearance in rat hippocampus reveals that repeated administration of p-methoxyamphetamine (PMA), but not 3,4-methylenedioxymethamphetamine (MDMA), leads to long-lasting deficits in serotonin transporter function. J. Neurochem. 100, 617–627.
Camp, M.C., Macpherson, K.P., Lederle, L., Graybeal, C., Gaburro, S., Debrouse, L.M., Ihne, J.L., Bravo, J.A., O'Connor, R.M., Ciocchi, S., Wellman, C.L., Lüthi, A., Cryan, J.F., Singewald, N., Holmes, A., 2012. Genetic strain differences in learned fear inhibition associated with variation in neuroendocrine, autonomic, and amygdala dendritic phenotypes. Neuropsychopharmacology 37, 1534–1547.
Carneiro, A.M., Airey, D.C., Thompson, B., Zhu, C.B., Lu, L., Chesler, E.J., Erikson, K.M., Blakely, R.D., 2009. Functional coding variation in recombinant inbred mouse lines reveals multiple serotonin transporter-associated phenotypes. Proc. Natl. Acad. Sci. 106, 2047–2052.
Chandana, S.R., Behen, M.E., Juhász, C., Muzik, O., Rothermel, R.D., Mangner, T.J., Chakraborty, P.K., Chugani, H.T., Chugani, D.C., 2005. Significance of abnormalities in developmental trajectory and asymmetry of cortical serotonin synthesis in autism. Int. J. Dev. Neurosci. 23, 171–182.
Christison, G.W., Ivany, K., 2006. Elimination diets in autism spectrum disorders: any wheat amidst the chaff? J. Dev. Behav. Pediatr. 27, S162–S171.
Coon, H., Dunn, D., Lainhart, J., Miller, J., Hamil, C., Battaglia, A., Tancredi, R., Leppert, M.F., Weiss, R., McMahon, W., 2005. Possible association between autism and variants in the brain-expressed tryptophan hydroxylase gene (TPH2). Am. J. Med. Genet. B Neuropsychiatr. Genet. 135B, 42–46.
Croonenberghs, J., Verkerk, R., Scharpe, S., Deboutte, D., Maes, M., 2005. Serotonergic disturbances in autistic disorder: L-5-hydroxytryptophan administration to autistic youngsters increases the blood concentrations of serotonin in patients but not in controls. Life Sci. 76, 2171–2183.
Daly, E.M., Deeley, Q., Ecker, C., Craig, M., Hallahan, B., Murphy, C., Johnston, P., Spain, D., Gillan, N., Brammer, M., Giampietro, V., Lamar, M., Page, L., Toal, F., Cleare, A., Surguladze, S., Murphy, D.G., 2012. Serotonin and the neural processing of facial emotions in adults with autism: an fMRI study using acute tryptophan depletion. Arch. Gen. Psychiatry 69, 1003–1013.
Daws, L.C., Gould, G.G., 2011. Ontogeny and regulation of the serotonin transporter: providing insights into human disorders. Pharmacol. Ther. 131, 61–79.
Deacon, R.M., 2006. Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. Nat. Protoc. 1, 122–124.
Delgado, P.L., 2006. Monoamine depletion studies: implications for antidepressant discontinuation syndrome. J. Clin. Psychiatry 67, 22–26.
Feder, A., Skipper, J., Blair, J.R., Buchholz, K., Mathew, S.J., Schwarz, M., Doucette, J.T., Alonso, A., Collins, K.A., Neumeister, A., Charney, D.S., 2011. Tryptophan depletion and emotional processing in healthy volunteers at high risk for depression. Biol. Psychiatry 69, 804–807.
Flood, Z.C., Engel, D.L., Simon, C.C., Negherbon, K.R., Murphy, L.J., Tamavimok, W., Anderson, G.M., Janušonis, S., 2012. Brain growth trajectories in mouse strains with central and peripheral serotonin differences: relevance to autism models. Neuroscience 210, 286–295.
Franklin, M., Bermudez, I., Murck, H., Singewald, N., Gaburro, S., 2012. Sub-chronic dietary tryptophan depletion – an animal model of depression with improved face and good construct validity. J. Psychiatr. Res. 46, 239–247.
Gabriele, S., Sacco, R., Persico, A.M., 2014. Blood serotonin levels in autism spectrum disorder: a systematic review and meta-analysis. Eur. Neuropsychopharmacol. 24, 919–929.
Giustarini, D., Dalle-Donne, I., Cavarra, E., Fineschi, S., Lungarella, G., Milzani, A., Rossi, R., 2006. Metabolism of oxidants by blood from different mouse strains. Biochem. Pharmacol. 71, 1753–1764.

Gould, G.G., Hensler, J.G., Burke, T.F., Benno, R.H., Onaivi, E.S., Daws, L.C., 2011. Density and function of central serotonin (5-HT) transporters, 5-HT1A and 5-HT2A receptors, and effects of their targeting on BTBR T+tf/J mouse social behavior. J. Neurochem. 116, 291–303.

Gould, G.G., Burke, T.F., Osorio, M.D., Smolik, C.M., Zhang, W.Q., Onaivi, E.S., Gu, T.T., DeSilva, M.N., Hensler, J.G., 2014. Enhanced novelty-induced corticosterone spike and upregulated serotonin 5-HT_{1A} and cannabinoid CB_1 receptors in adolescent BTBR mice. Psychoneuroendocrinology 39, 158–169.

Hattori, T., Kanno, K., Nagasawa, M., Nishimori, K., Mogi, K., Kikusui, T., 2014. Impairment of interstrain social recognition during territorial aggressive behavior in oxytocin receptor-null mice. Neurosci. Res. (in press).

Henry, C.A., Shervin, D., Neumeyer, A., Steingard, R., Spybrook, J., Choueiri, R., Bauman, M., 2009. Retrial of selective serotonin reuptake inhibitors in children with pervasive developmental disorders: a retrospective chart review. J. Child. Adolesc. Psychopharmacol. 19, 111–117.

Herndon, A.C., DiGuiseppi, C., Johnson, S.L., Leiferman, J., Reynolds, A., 2009. Does nutritional intake differ between children with autism spectrum disorders and children with typical development? J. Autism Dev. Disord. 39, 212–222.

Hjiej, H., Doyen, C., Couprie, C., Kaye, K., Contejean, Y., 2008. Substitutive and dietetic approaches in childhood autistic disorder: interests and limits. Encephale 34, 496–503.

Hill, J.E., Makky, K., Shrestha, L., Hillard, C.J., Gasser, P.J., 2011. Natural & synthetic corticosteroids inhibit uptake 2-mediated transport in CNS neurons. Physiol. Behav. 104, 306–311.

Hohmann, C.F., Walker, E.M., Boylan, C.B., Blue, M.E., 2007. Neonatal serotonin depletion alters behavioral responses to spatial change and novelty. Brain Res. 1139, 163–177.

Hollander, E., Soorya, L., Chaplin, W., Anagnostou, E., Taylor, B.P., Ferretti, C.J., Wasserman, S., Swanson, E., Settipani, C., 2012. A double-blind placebo-controlled trial of fluoxetine for repetitive behaviors and global severity in adult autism spectrum disorders. Am. J. Psychiatry 169, 292–299.

Horton, R.E., Apple, D.M., Owens, W.A., Baganz, N.L., Cano, S., Mitchell, N.C., Vitela, M., Gould, G.G., Koek, W., Daws, L.C., 2013. Decynium-22 enhances SSRI-induced antidepressant-like effects in mice: uncovering novel targets to treat depression. J. Neurosci. 33, 10534–10543.

Hoshino, Y., Yamamoto, T., Kaneko, M., Tachibana, R., Watanabe, M., Ono, Y., Kumashiro, H., 1984. Blood serotonin and free tryptophan concentration in autistic children. Neuropsychobiology 11, 22–27.

Hoshino, Y., Yamamoto, T., Kaneko, M., Kumashiro, H., 1986. Plasma free tryptophan concentration in autistic children. Brain Dev. 8, 424–427.

Hyman, S.L., Stewart, P.A., Schmidt, B., Cain, U., Lemcke, N., Foley, J.T., Peck, R., Clemons, T., Reynolds, A., Johnson, C., Handen, B., James, S.J., Courtney, P.M., Molloy, C., Ng, P.K., 2012. Nutrient intake from food in children with autism. Pediatrics 130, S145–S153.

Jans, L.A., Korte-Bouws, G.A., Korte, S.M., Blokland, A., 2010. The effects of acute tryptophan depletion on affective behaviour and cognition in Brown Norway and Sprague Dawley rats. J. Psychopharmacol. 24, 605–614.

Janušonis, S., 2008. Origin of the blood hyperserotonemia of autism. Theor. Biol. Med. Model. 5, 10.

John, A.M., Bell, J.M., 1976. Amino acid requirements of the growing mouse. J. Nutr. 106, 1361–1367.

Johnson, C.R., Turner, K., Stewart, P.A., Schmidt, B., Shui, A., Macklin, E., Reynolds, A., James, J., Johnson, S.L., Manning, C.P., Hyman, S.L., 2014. Relationships between feeding problems, behavioral characteristics and nutritional quality in children with ASD. J. Autism Dev. Disord. 44, 2175–2184.

Kalueff, A.V., Keisala, T., Minasyan, A., Kuuslahti, M., Miettinen, S., Tuohimaa, P., 2006. Behavioural anomalies in mice evoked by "Tokyo" disruption of the Vitamin D receptor gene. Neurosci. Res. 54, 254–260.

Kane, M.J., Angoa-Peréz, M., Briggs, D.I., Sykes, C.E., Francescutti, D.M., Rosenberg, D.R., Kuhn, D.M., 2012. Mice genetically depleted of brain serotonin display social impairments, communication deficits and repetitive behaviors: possible relevance to autism. PLoS One 7, e48975.

Kidd, P.M., 2002. Autism, an extreme challenge to integrative medicine: Part 2. Medical management. Altern. Med. Rev. 7, 472–499.

Kałuzna-Czaplinska, J., Michalska, M., Rynkowski, J., 2010. Determination of tryptophan in urine of autistic and healthy children by gas chromatography/mass spectrometry. Med. Sci. Monit. 16, CR488–CR492.

Kumar, B., Prakash, A., Sewal, R.K., Medhi, B., Modi, M., 2012. Drug therapy in autism: a present and future perspective. Pharmacol. Rep. 64, 1291–1304.

Lakhan, S.E., Vieira, K.F., 2008. Nutritional therapies for mental disorders. Nutr. J. 7, 2.

Lam, K.S., Aman, M.G., Arnold, L.E., 2006. Neurochemical correlates of autistic disorder: a review of the literature. Res. Dev. Disabil. 27, 254–289.

Macbeth, A.H., Lee, H.J., Edds, J., Young, W.S., 2009. Oxytocin and the oxytocin receptor underlie intrastrain, but not interstrain, social recognition. Genes. Brain Behav. 8, 558–567.

Madden, A.M., Zup, S.L., 2014. Effects of developmental hyperserotonemia on juvenile play behavior, oxytocin and serotonin receptor expression in the hypothalamus are age and sex dependent. Physiol. Behav. 128, 260–269.

Makkonen, I., Riikonen, R., Kokki, H., Airaksinen, M.M., Kuikka, J.T., 2008. Serotonin and dopamine transporter binding in children with autism determined by SPECT. Dev. Med. Child. Neurol. 50, 593–597.

Marcason, W., 2009. What is the current status of research concerning use of a gluten-free, casein-free diet for children diagnosed with autism? J. Am. Diet. Assoc. 109, 572.

Marí-Bauset, S., Zazpe, I., Mari-Sanchis, A., Llopis-González, A., Morales-Suárez-Varela, M., 2014. Food Selectivity in autism spectrum disorders: systematic review. J. Child. Neurol. 29, 1554–1561.

McDougle, C.J., Naylor, S.T., Goodman, W.K., Volkmar, F.R., Cohen, D.J., Price, L.H., 1993. Acute tryptophan depletion in autistic disorder: a controlled case study. Biol. Psychiatry 33, 547–550.

McDougle, C.J., Naylor, S.T., Cohen, D.J., Aghajanian, G.K., Heninger, G.R., Price, L.H., 1996. Effects of tryptophan depletion in drug-free adults with autistic disorder. Arch. Gen. Psychiatry 53, 993–1000.

McNamara, I.M., Borella, A.W., Bialowas, L.A., Whitaker-Azmitia, P.M., 2008. Further studies in the developmental hyperserotonemia model (DHS) of autism: social, behavioral and peptide changes. Brain Res. 1189, 203–214.

Moy, S.S., Nadler, J.J., Young, N.B., Perez, A., Holloway, L.P., Barbaro, R.P., Barbaro, J.R., Wilson, L.M., Threadgill, D.W., Lauder, J.M., Magnuson, T.R., Crawley, J.N., 2007. Mouse behavior tasks relevant to autism: phenotypes of 10 inbred strains. Behav. Brain Res. 176, 4–20.

Nakamura, K., Hasegawa, H., 2009. Production and peripheral roles of 5-HTP, a precursor of serotonin. Int. J. Tryptophan Res. 2, 37–43.

Nakamura, K., Sekine, Y., Ouchi, Y., Tsujii, M., Yoshikawa, E., Futatsubashi, M., Tsuchiya, K.J., Sugihara, G., Iwata, Y., Suzuki, K., Matsuzaki, H., Suda, S., Sugiyama, T., Takei, N., Mori, N., 2010. Brain serotonin and dopamine transporter bindings in adults with high-functioning autism. Arch. Gen. Psychiatry 67, 59–68.

Naushad, S.M., Jain, J.M., Prasad, C.K., Naik, U., Akella, R.R., 2013. Autistic children exhibit distinct plasma amino acid profile. Indian J. Biochem. Biophys. 50, 474–478.

National Research Council, 1995. Nutrient requirements of the mouse. In: Nutrient Requirements of Laboratory Animals, fourth ed. National Academies Press, Washington, DC, pp. 80–102.

Patrick, R.P., Ames, B.N., 2014. Vitamin D hormone regulates serotonin synthesis. Part 1: relevance for autism. FASEB J. 28, 2398–2413.

Politte, L.C., Henry, C.A., McDougle, C.J., 2014. Psychopharmacological interventions in autism spectrum disorder. Harv. Rev. Psychiatry 22, 76–92.

Ricci, M.R., Ulman, E.A., 2005. Laboratory animal diets: a critical part of your in vivo research. Anim. Lab. News 4, 1–6.

Rubin, D.H., Althoff, R.R., Ehli, E.A., Davies, G.E., Rettew, D.C., Crehan, E.T., Walkup, J.T., Hudziak, J.J., 2013. Candidate gene associations with withdrawn behavior. J. Child. Psychol. Psychiatry 54, 1337–1345.

Ruggeri, B., Sarkans, U., Schumann, G., Persico, A.M., 2014. Biomarkers in autism spectrum disorder: the old and the new. Psychopharmacology (Berl) 231, 1201–1216.

Schwartz, C.E., 2014. Aberrant tryptophan metabolism: the unifying biochemical basis for autism spectrum disorders? Biomark. Med. 8, 313–315.

Silverman, J.L., Yang, M., Lord, C., Crawley, J.N., 2010. Behavioural phenotyping assays for mouse models of autism. Nat. Rev. Neurosci. 11, 490–502.

Temme, S.J., Bell, R.Z., Pahumi, R., Murphy, G.G., 2014. Comparison of inbred mouse substrains reveals segregation of maladaptive fear phenotypes. Front. Behav. Neurosci. 8, 282.

Tanoue, K., Matsui, K., Takamasu, T., 2012. Fried-potato diet causes vitamin A deficiency in an autistic child. JPEN J. Parenter. Enter. Nutr. 36, 753–755.

Terada, Y., Okura, Y., Kikusui, T., Takenaka, A., 2011. Dietary vitamin E deficiency increases anxiety-like behavior in juvenile and adult rats. Biosci. Biotechnol. Biochem. 75, 1894–1899.

Toker, L., Amar, S., Bersudsky, Y., Benjamin, J., Klein, E., 2010. The biology of tryptophan depletion and mood disorders. Isr. J. Psychiatry Relat. Sci. 47, 46–55.

Uchida, S., Kitamoto, A., Umeeda, H., Nakagawa, N., Masushige, S., Kida, S., 2005. Chronic reduction in dietary tryptophan leads to changes in the emotional response to stress in mice. J. Nutr. Sci. Vitaminol. 51, 175–181.

Unwin, L.M., Maybery, M.T., Wray, J.A., Whitehouse, A.J., 2013. A "bottom-up" approach to aetiological research in autism spectrum disorders. Front. Hum. Neurosci. 7, 606.

van Donkelaar, E.L., Blokland, A., Lieben, C.K., Kenis, G., Ferrington, L., Kelly, P.A., Steinbusch, H.W., Prickaerts, J., 2010. Acute tryptophan depletion in C57BL/6 mice does not induce central serotonin reduction or affective behavioural changes. Neurochem. Int. 56, 21–34.

van Donkelaar, E.L., Blokland, A., Ferrington, L., Kelly, P.A., Steinbusch, H.W., Prickaerts, J., 2011. Mechanism of acute tryptophan depletion: is it only serotonin? Mol. Psychiatry 16, 695–713.

Veenstra-VanderWeele, J., Muller, C.L., Iwamoto, H., Sauer, J.E., Owens, W.A., Shah, C.R., Cohen, J., Mannangatti, P., Jessen, T., Thompson, B.J., Ye, R., Kerr, T.M., Carneiro, A.M., Crawley, J.N., Sanders-Bush, E., McMahon, D.G., Ramamoorthy, S., Daws, L.C., Sutcliffe, J.S., Blakely, R.D., 2012. Autism gene variant causes hyperserotonemia, serotonin receptor hypersensitivity, social impairment and repetitive behavior. Proc. Natl. Acad. Sci. 109, 5469–5474.

West, L., Waldrop, J., Brunssen, S., 2009. Pharmacologic treatment for the core deficits and associated symptoms of autism in children. J. Pediatr. Health Care 23, 75–89.

Whitehouse, A.J.O., Stanley, F.J., 2013. Is autism one or multiple disorders? Med. J. Aust. 28, 302–303.

Williams, K., Brignell, A., Randall, M., Silove, N., Hazell, P., 2013. Selective serotonin reuptake inhibitors (SSRIs) for autism spectrum disorders. Cochrane DB Syst. Rev. 8, CD004677.

Yang, C.J., Tan, H.P., Du, Y.J., 2014. The developmental disruptions of serotonin signaling may be involved in autism during early brain development. Neuroscience 267C, 1–10.

Yang, M., Silverman, J.L., Crawley, J.N., 2011. Automated three-chambered social approach task for mice. Curr. Protoc. Neurosci. 56, 8.26.1–8.26.16.

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Ontogeny of SERT expression and antidepressant-like response to escitalopram in wild-type and SERT mutant mice.

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List of abbreviations: maximal binding (Bmax), half-maximally effective dose (ED_{50}), maximal effect (Emax), forced swim test (FST), affinity (K_d), postnatal day (P), serotonin (5-HT), selective serotonin reuptake inhibitor (SSRI), serotonin transporter (SERT), tail suspension test (TST), ventromedial hypothalamus (VMH).

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ABSTRACT

Depression is a disabling affective disorder for which the majority of patients are not effectively treated. This problem is exacerbated in children and adolescents for whom only two antidepressants are approved, both selective serotonin reuptake inhibitor (SSRIs). Unfortunately SSRIs are often less effective in juveniles than in adults; however, the mechanism(s) underlying age-dependent responses to SSRIs is unknown. To this end we compared antidepressant-like response to the SSRI escitalopram using the tail suspension test and saturation binding of [^{3}H]citalopram to the serotonin transporter (SERT), the primary target of SSRIs, in juvenile (21 days post-natal [P21]), adolescent (P28) and adult (P90) wild-type (SERT+/+) mice. In addition, to model individuals carrying low expressing SERT variants, we studied mice with reduced SERT expression (SERT+/-) or lacking SERT (SERT-/-). Maximal antidepressant-like effects were less in P21 mice relative to P90 mice. This was especially apparent in SERT+/- mice. However, the potency for escitalopram to produce antidepressant-like effects in SERT+/+ and SERT+/- mice was greater in P21 and P28 mice than adults. SERT expression increased with age in terminal regions and decreased with age in cell body regions. Binding affinity values did not change as a function of age or genotype. As expected, in SERT-/- mice escitalopram produced no behavioral effects, and there was no specific [^{3}H]citalopram binding. These data reveal age- and genotype-dependent shifts in the dose-response for escitalopram to produce antidepressant-like effects, which vary with SERT expression, and may contribute to the limited therapeutic response to SSRIs in juveniles and adolescents.

INTRODUCTION

Depression is a disabling affective disorder for which the majority of patients are not effectively treated. This problem is exacerbated in juveniles and adolescents by limited therapeutic options (Bylund and Reed, 2007). Only two selective serotonin (5-HT) reuptake inhibitor (SSRI) antidepressants, escitalopram and fluoxetine, have US Food and Drug Administration approval as treatments for pediatric depression. SSRIs act on the serotonergic system by blocking the 5-HT transporter (SERT) (SLC6A4), the high affinity clearance mechanism for extracellular 5-HT. The resultant increase in extracellular 5-HT is thought to trigger therapeutic downstream effects. However, escitalopram and fluoxetine are often less effective in treating symptoms of depression in juveniles and adolescents than in adults (Tsapakis et al., 2008; Hetrick et al., 2009 & 2010). Further, in adolescents certain SERT gene variants are associated with increased risk for developing SSRI-resistant depression after stressful life events, triggering a lifelong struggle with the disorder (Serretti et al., 2007; Petersen et al., 2012). Given the high incidence of depression in adolescents, 4-8% of the US population, and the fact that suicide is the third most likely cause of death in this age group, there is a distinct need to uncover mechanism(s) limiting the therapeutic benefits of SSRIs in young patients (Kessler et al., 2001; Bujoreanu et al., 2011). A better understanding of the age-dependency of SSRI efficacy is therefore an important first step toward improving therapeutics for pediatric depression.

Rodents are indispensable tools for studying the behavioral and neurochemical effects of antidepressants. However, most studies to date have focused on antidepressant-like behavior in adult rodents and have largely ignored use of juveniles and adolescents.

One study reported that SSRIs reduced immobility time in the forced swim test (FST), an index of antidepressant-like action, in juvenile (post-natal day [P] 21) and adolescent (P28-P35) rats (Reed et al., 2008). Recently we discovered differences in behavioral responses to SSRIs in juvenile mice. We used escitalopram at a dose of 10 mg/kg, which is known to produce maximal antidepressant-like effects in adult mice in the tail suspension test (TST), an assay commonly used to assess antidepressant-like activity in mice (Cryan et al., 2005; O'Leary et al., 2007). At this dose the antidepressant-like effect of escitalopram was less in juvenile mice than in adult mice (Mitchell et al*.,* 2013), a finding consistent with clinical reports. The present study expands upon this initial finding by examining the dose-dependency of escitalopram's antidepressant-like effects in juvenile (P21), adolescent (P28) and adult (P90) mice. In addition, because individuals carrying low expressing variants of SERT are often reported to benefit less from treatment with SSRIs than individuals who do not harbor such gene variants, we studied mice with reduced SERT expression (SERT+/-) or lacking SERT (SERT-/-) and compared them to wild-type mice (SERT+/+).

In murine models, serotonergic neuronal innervation, dendritic connections and extracellular 5-HT concentration have been shown to be similar to adults by P21 (Loizou et al., 1970; Wallace and Lauder, 1983; Miranda-Contreras et al., 1998). These studies suggest that some aspects of the central serotonergic system are functionally developed in juveniles; however, a developmental lag in SERT expression or function could limit the therapeutic benefit of SSRIs. In rats, SERT expression reaches adult levels between birth and P21 with expression patterns varying in different brain regions (Bylund and Reed, 2007; Galineau et al., 2004; Moll et al., 2000; Slotkin et al., 2008).

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However, these studies did not evaluate possible developmental changes in SERT affinity (K_d). Recently we discovered that SERT binding properties in juvenile (P21) and adolescent (P28) mice may differ from adults. While maximal binding (Bmax) of the SERT-selective ligand, [^{3}H]citalopram, in hippocampal membranes revealed no differences among ages, variance in K_d for P21 mice was significantly greater than in older mice (Mitchell et al., 2013), suggesting that functionality of SERT may be at a developmentally dynamic transitional stage. To better characterize age- and brain region-dependent changes in SERT function, the present study used quantitative autoradiography, an anatomically more sensitive assay, to examine [^{3}H]citalopram binding properties and their relation to behavior in the TST.

MATERIALS AND METHODS

Animals

Male and female SERT wild-type (SERT+/+), heterozygote (SERT+/-) or homozygote knockout (SERT-/-) mice (backcrossed to C57BL/6J for >10 generations) were used for all experiments. Colony founders were provided by Dr. Dennis Murphy (National Institute of Mental Health), and were bred and identified as previously described (Bengel et al., 1998). P21 (juvenile), P28 (adolescent), and P90-P100 (adult) mice (Spear, 2000) were used. Mice of each genotype were generated by crossing male and female SERT+/- mice. Animals were housed in a temperature-controlled (24°C) vivarium maintained on a 12/12-hr light/dark cycle (lights on at 7:00 am) in plastic cages (29cm x 18cm x 13 cm) containing rodent bedding (Sani-chips, Harlan Teklad, Madison, WI, USA) with free access to food (irradiated rodent sterilizable diet, Harlan Teklad, Madison, WI, USA) and water. After weaning on P28, mice were housed in groups of five with same-sex peers. TST experiments were conducted prior to weaning in mice aged P21 and P28. All procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council 1996), and with the approval of the Institutional Animal Care and Use Committee, The University of Texas Health Science Center at San Antonio.

Tail suspension test

The TST was conducted as originally described by Steru et al. (1985) (for review see [Castagné et al., 2011]). Naïve mice were moved from the colony room to testing room

and allowed a 1-2 hour acclimation period. All experiments were conducted between 12:00 noon and 5:00 pm. All mice received saline vehicle intraperitoneally (i.p.) one hour before testing, followed 30 minutes (min) later by a subcutaneous (s.c.) injection of either escitalopram (0.1, 0.32, 1.0, 3.2 or 10.0 mg/kg) or saline vehicle (control condition). This drug administration protocol was selected in order to be consistent with our previously published procedure (Baganz et al., 2008; Horton et al., 2013; Mitchell et al., 2015). In addition, administration of antidepressant 30 min before testing is standard (Steru et al., 1985; Holmes et al., 2002; Ripoll et al., 2003). Immediately before testing, the distal portion of the tail was fastened to a flat aluminum (2 x 0.3 x 10 cm) bar using adhesive tape placed at a 90° angle to the longitudinal axis of the mouse tail, with 3-4 cm between the base of the mouse tail and the aluminum bar. A hole opposite the taped end of the bar was used to secure the bar to a hook on the ceiling of a visually isolated white box (40 x 40 x 40 cm). Each mouse was suspended by its tail for 6 min, allowing the ventral surface and the front and hind limbs to be recorded using a digital video camera facing the testing box. Total immobility time was measured (in seconds (s)) during the 6 min time period. Immobility was defined as the absence of initiated movements, and included passive swaying of the body. A mouse was excluded from the study if it climbed and held its tail or the aluminum bar for a period of 3 s or longer. Immobility was scored twice from the videos by observers who were blind to the treatments. Each mouse was tested only once, and all mice were randomly assigned to treatments.

Drugs

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Escitalopram (*s*-citalopram or (*S*)-1[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile) oxalate [Sigma-Aldrich (St. Louis, MO, USA)] was dissolved in physiological saline and injected s.c. at doses expressed as base weight per kilogram body weight. The injection volume was 10 ml/kg. Drug doses were chosen that ranged from subthreshold (0.32 mg/kg) to maximally effective (10 mg/kg) in adult SERT+/+ mice.

Autoradiography

Serotonin transporter (SERT) saturation binding was assessed in mouse brain by quantitative autoradiography using the SERT-selective ligand [^{3}H]citalopram and methods adapted from D'Amato et al., (1987). Naïve mice were killed by decapitation and brains rapidly removed and frozen on powdered dry ice before being stored at -80 °C until sectioned for quantitative autoradiography. Brains were brought to -20 °C in a cryostat (Leica CM 1850, Meyer Instruments, Houston, TX) and coronal sections (20 µm) were collected at the level of plate 12 (prefrontal cortex), plate 47 (hippocampal subregions [CA1, 2, 3 and dentate gyrus], parietal cortex, amygdala and ventromedial hypothalamus) and plate 64 (raphe nuclei) according to Paxinos and Franklin's mouse brain atlas (1997). Sections were thaw mounted onto gelatin-coated microscope slides, vacuum desiccated for 18-24 h at 4 °C and stored at -80 °C until use. In preparation for binding, sections were thawed, then pre-incubated for 1 h in a 50 mM Tris-HCl, 120 mM NaCl, and 5 mM KCl pH 7.4 at room temperature (~ 24°C). Incubation was carried out in slide mailers (VWR, USA) filled with 10 ml of the same buffer containing [^{3}H]citalopram at concentrations of 0.32, 0.56, 1.0, 3.2, or 5.6 nM for 1 h at room temperature. Non-specific binding was defined by 20 µM sertraline (Pfizer, Groton CT)

and was ~11-23 % total binding in low binding regions (i.e. parietal cortex) and ~5-9 % total binding in high binding regions (i.e. dorsal raphe). The incubation was terminated by two 10 min washes in 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl pH 7.4 at 4 °C, followed by a 5 s dip in de-ionized water at 4 °C. Slides were dried on a slide warmer for 20 min. [^{3}H]citalopram labeled sections were exposed to Carestream Biomax MR film for 6 weeks, along with tritium standards (American Radiolabeled Chemicals, St Louis, MO). Films were developed in a film processor (AFP imaging, Elmsford, NY). Autoradiogram images were captured on a digital image system: 12-digital bit camera (CFW-1612M, Scion Corp., Frederick, MD), Nikon Lens, Northern Lights Illuminator and Kaiser RS1 copy stand (all from InterFocus Imaging Ltd., Linton, England) and were calibrated and measured with NIH image J public access shareware (http//rsb.info.nih.gov/ij/download) on a MacBook (OS 10).

Data analysis

Statistical analyses were performed using Prism 6.0 (GraphPad, San Diego, CA, USA).

TST: Under vehicle conditions, we previously found that time spent immobile varies by age and SERT genotype (Mitchell et al., 2013; 2015). Because similar findings were obtained here (Figure I.A), dose-response data were expressed as percent from vehicle control (Figure I.B-D) and analyzed by two-factor ANOVA (age, drug dose) followed by Tukey's and Dunnett's multiple comparisons tests. Within each genotype and age group there were no significant differences in drug-induced immobility between males and females (main effect of sex: $P \geq 0.11$; interaction between sex and dose, $P \geq 0.10$) (data not shown); thus, data for both sexes were pooled. Maximal effect (Emax) and half-maximally effective dose (ED_{50}) values, derived from data shown in Figure I.B,C are

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shown in Figure I.E,F. All data are expressed as mean ± standard error of the mean (SEM), except ED_{50} values, which are expressed as the mean. $P < 0.05$ was considered statistically significant.

Emax was defined as the greatest observed percent change in immobility from the saline control condition. To calculate ED_{50} values, time spent immobile was expressed as percent of vehicle control, and the linear portion of the dose-response curves was analyzed by log-linear regression of data from individual subjects, with the following equation: effect = slope X log(dose) + intercept, using methods detailed elsewhere (Koek et al., 2009). Maximal effects were analyzed using a two-factor ANOVA (age, genotype) followed by Tukey's multiple comparisons test.

Quantitative Autoradiography: Nonspecific binding was subjected to unweighted linear regression and subtracted from total [^{3}H]citalopram binding to give specific binding. Specific [^{3}H]citalopram binding data were submitted to unweighted non-linear regression and the saturation binding isotherm fitted according to a one site model: $Y = Bmax * X / (K_d + X)$. Mean Bmax and K_d values were analyzed using a two-factor (age, genotype) ANOVA (Figure IV), with Tukey's *post hoc* test for multiple comparisons. Within genotype-, age- and brain region-matched groups there were no statistically-significant differences in Bmax and K_d values for [^{3}H]citalopram binding between males and females; thus, data from both sexes were pooled ($P \geq 0.051$) (data not shown). All data are expressed as mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

Correlations: As shown in Figures V.A-F, Pearson's correlation was used to examine the relationship between the Emax for escitalopram to reduce immobility time in the

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TST, values taken from Figure I.F, and maximal specific [^{3}H]citalopram binding, values taken from Figure IV.A-F. All data are expressed as mean ± standard error of the mean (SEM).

RESULTS

Maximal effect and potency of escitalopram to reduce immobility time in the tail suspension test is age- and SERT- genotype-dependent.

Under control (saline-injected) conditions (Figure I.A), basal immobility varied in an age- and genotype-dependent manner [age vs SERT-genotype interaction: $F(4,169) = 7.35$, $P < 0.0001$). Baseline immobility increased with age SERT+/- mice, but not in SERT+/+ or SERT-/- mice (Figure I.A). At P21, differences among the genotypes were not statistically significant. At P28 and P90, SERT-/- mice showed less immobility than SERT+/+ mice. At P90, SERT-/- mice showed less immobility than at P21; in contrast, SERT+/- mice showed more immobility at P90 than at P21.

In SERT+/+ mice, escitalopram reduced immobility time in the TST in all age groups [$F(4, 299) = 56.3$, $P < 0.0001$] (Figure I.B). Overall, P21 and P28 mice spent less time immobile than P90 mice following escitalopram [$F(2, 299) = 7.86$, $P = 0.0005$]. In addition, escitalopram dose interacted with age [$F(8, 299) = 3.39$, $P = 0.001$]: at low doses, P21 and P28 mice showed less immobility than mice aged P90. P21 SERT+/+ mice spent less time immobile than P90 mice following 1.0 and 3.2 mg/kg doses of escitalopram ($P = 0.013$; $P = 0.012$), and P28 SERT+/+ mice spent significantly less time immobile than P90 mice following 0.32, 1.0 and 3.2 mg/kg doses of escitalopram ($P < 0.0001$; $P = 0.013$; $P = 0.005$, respectively). There was no significant difference in time spent immobile among ages following 10 mg/kg escitalopram, though there was a trend for P21 mice to spend more time immobile than P90 mice ($P = 0.11$). The lowest effective dose for P28 was 0.32 mg/kg escitalopram ($P = 0.0004$), while 1.0 mg/kg was the lowest for P21 ($P < 0.0001$) and P90 mice ($P = 0.008$).

As for SERT+/+ mice, escitalopram reduced immobility in all age groups of SERT+/- mice [$F(4, 250) = 37.4$, $P < 0.0001$] and its effects interacted with age [$F(8,250) = 2.60$, $P = 0.0095$] (Figure I.C). P21 SERT+/- mice spent significantly less time immobile than P90 SERT+/- mice following 0.32 mg/kg escitalopram ($P = 0.01$), but significantly more time immobile following 10 mg/kg escitalopram ($P = 0.006$). The lowest effective dose for P21 and P28 SERT+/- mice was 0.32 mg/kg ($P < 0.001$; $P = 0.014$), while the lowest effective dose for P90 SERT+/- mice was 1.0 mg/kg ($P < 0.001$).

Because previous studies have shown that SSRIs do not produce antidepressant-like effects in SERT-/- mice (Holmes et al., 2002), we evaluated only the 10 mg/kg dose of escitalopram, which is maximally effective in P90 SERT+/+ and SERT+/- mice. As expected, there was no effect of drug [$F(1,115) = 0.86$, $P = 0.42$], age [$F(2,115) = 0.86$, $P = 0.42$], or interaction [$F(2,115) = 0.86$, $P = 0.42$] (Figure I.D). There were no significant differences in immobility among ages and between vehicle and 10 mg/kg escitalopram.

ED_{50} and Emax values for escitalopram to reduce immobility time in SERT+/+ and SERT+/- mice are summarized in Figure I.E, F (SERT-/- mice were not included in the analysis because escitalopram did not alter immobility time in these mice). The ED_{50} of escitalopram to inhibit immobility varied as a function of age [SERT+/+: $F(2,191) = 19.45$, $P < 0.001$; SERT+/-: $F(2, 156) = 4.18$, $P = 0.017$]; escitalopram more potently inhibited immobility in P21 and P28 mice than in P90 mice for both genotypes [SERT+/+: $F(1, 133) = 15.7$, $P < 0.001$; $F(1,137) = 34.7$, $P < 0.001$; SERT+/-: [$F(1, 104) = 6.24$, $P = 0.014$; $F(1, 110) = 5.7$; $P = 0.019$] (Figure I.E). Within-age comparisons showed no potency difference of escitalopram to reduce immobility in SERT+/+ and

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SERT+/- mice aged P21 and P28. However, at P90 the ED_{50} value was significantly lower in SERT+/- mice than in SERT+/+ mice [$F(1, 138) = 8.35$; $P = 0.005$].

In SERT+/+ and SERT +/- mice, Emax values for escitalopram were smaller in young mice than in adult (P90) mice [$F(2, 103) = 7.59$; $P < 0.001$] (Figure I.F). Genotype had no effect on Emax values [$F(1,103) = 0.55$, $P = 0.46$], nor was there an age by genotype interaction [$F(2,103) = 7.59$, $P = 0.86$]. Emax values in SERT+/+ and SERT+/- mice aged P21 were smaller than those for genotype matched P90 mice ($P = 0.047$; $P = 0.008$, respectively). No significant difference was found between P28 and P90 Emax values in SERT+/+ and SERT+/- mice ($P = 0.12$; $P = 0.10$, respectively).

In sum, for SERT+/+ and SERT+/- mice the ED_{50} value for escitalopram to decrease immobility time was lower in P21 and P28 mice than in P90 mice, suggesting that escitalopram is more potent in juvenile and adolescent mice than in adult mice. In contrast, Emax values were smaller in young mice than in P90 mice, suggesting that escitalopram is less efficacious in juvenile and adolescent mice than in adult mice.

SERT expression increases with age in select serotonin terminal regions, and decreases with age in cell body regions.

Maximal specific [^{3}H]citalopram binding (Bmax) values and K_d values derived from curve fitting were analyzed separately for each brain region with a two-factor ANOVA (age, genotype) (Figure II, III & IV; Table I). As expected, [^{3}H]citalopram binding in SERT+/- mice was less than that in SERT+/+ mice and there was no [^{3}H]citalopram binding detected in SERT-/- mice (Figure II & III).

Terminal Regions:

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Bmax for [^{3}H]citalopram binding increased with age in CA1, CA2, and dentate gyrus [F(2,44) = 9.84, P < 0.001; F(2, 44) = 9.42, P < 0.001; F(2,44) = 7.17, P = 0.002]; however, age did not significantly influence Bmax for [^{3}H]citalopram binding in CA3 [F(2, 44) = 2.80, P = 0.071] (Figure IV.A-D). As expected, Bmax values for [^{3}H]citalopram binding in SERT+/- mice were lower than those in SERT+/+ mice [F(1,44) = 182, p < 0.001; F(1,44) = 206, P < 0.001; F(1,44) = 240, P < 0.001; F(1,44) = 132, P < 0.001; for CA1, CA2, CA3 and dentate gyrus respectively]. No interactions were found between age and SERT-genotype in the CA1, CA2, CA3 regions of hippocampus or dentate gyrus [F(2,44) = 0.24, P = 0.79; F(2,44) = 0.66, P = 0.52; F(2,44) = 0.13, P = 0.88; F(2,44) = 0.56, P = 0.58]. Among age comparisons showed Bmax values to be smaller in P21 SERT+/+ and P28 SERT+/+ mice than in P90 SERT+/+ mice in CA2 and dentate gyrus (P < 0.01; P < 0.05; for P21 and P28 mice). In CA1, Bmax values for [^{3}H]citalopram binding were smaller in P21 SERT+/+ and P21 SERT+/- mice than in the SERT genotype-matched P90 group (P < 0.01; P < 0.05; for SERT+/+ and SERT+/- mice respectively).

Effects of age on Bmax for [^{3}H]citalopram binding in either the prefrontal cortex, parietal cortex or ventromedial hypothalamus (VMH) were not statistically significant [F(2,44) = 0.68, P = 0.51; F(2,44) = 2.858, P = 0.07; F(2,44) = 2.16, P = 0.13, respectively] (Table I), except that Bmax in the parietal cortex of SERT+/+ mice was lower in P21 than in P90 mice. Bmax values for [^{3}H]citalopram binding in SERT+/+ mice were higher than those for SERT+/- mice in the prefrontal cortex, parietal cortex and VMH [F(1,44) = 154, P < 0.01; F(1,44) = 64.9, P < 0.01; F(1,44) = 90.27, P < 0.01 respectively], and no

interaction was found between age and SERT-genotype [F(2,44) = 0.32, P = 0.73; F(2,44) = 0.47, P = 0.63; F(2,44) = 0.61, P = 0.55 respectively].

Bmax for [^{3}H]citalopram binding increased with age in the amygdala [F(2,44) = 5.17, P < 0.01] (Table I). As expected, lower [^{3}H]citalopram binding was found in amygdala of SERT+/- mice compared with SERT+/+ mice [F(1,44) = 112.7, P < 0.01]. There was no interaction between age and SERT-genotype [F(2,44) = 0.97, P = 0.39]. Among age comparisons showed Bmax for [^{3}H]citalopram binding in amygdala to be lower in P28 SERT+/- mice than in P90 SERT+/- mice (P = 0.046) (Table I).

There was no effect of age or genotype on K_d values for [^{3}H]citalopram binding in any terminal region (data not shown, P ≥ 0.06), values ranged from 0.6 - 1.4 nM [^{3}H]citalopram.

Cell body regions:

Bmax for [^{3}H]citalopram binding significantly decreased with age in the median raphe nucleus [F(2,44) = 10.3, P < 0.01] (Figure IV.F). In the dorsal raphe, a statistically non-significant trend showed lower Bmax values in younger mice than in older mice [F(2,44) = 2.46, P = 0.098] (Figure IV.E). In both cell body regions, higher Bmax values were observed in SERT+/+ mice than in SERT+/- mice [F(1,44) = 86.7, P < 0.01; F(1,44) = 78.7, P < 0.01; median and dorsal raphe respectively]. For both median or dorsal raphe, no interactions between SERT-genotype and age were observed [F(2,44) = 0.1, P = 0.91; F(2,44) = 0.3, P = 0.74]. Among-age comparisons in the median and dorsal raphe of SERT+/+ mice showed greater Bmax values in P21 than in P90 mice (P <

0.01, P = 0.04). Similarly, greater Bmax values were found in the median raphe of P21 SERT+/- mice than in P90 SERT+/- mice (P < 0.01).

There were no statistically significant effects of age or SERT-genotype on K_d values for [^{3}H]citalopram binding (data not shown, P ≥ 0.051). Values ranged from 0.54 – 0.85 nM [^{3}H]citalopram.

Relationship between antidepressant-like effects of escitalopram in the tail suspension test and [^{3}H]citalopram binding in hippocampus and raphe nuclei.

Terminal regions:

Figure V.A-D shows a positive relation between Emax values (i.e. antidepressant-like response) for escitalopram and Bmax values for [^{3}H]citalopram binding in hippocampal subregions as a function of age and SERT-genotype. In both SERT+/+ and SERT+/- mice this positive relation can be adequately described by a straight line in each of the hippocampal regions (r ≥ 0.80 to 0.99, Pearson's correlation) (Figure V.A-D).

Cell body regions:

Figure V.E-F shows a negative relation between Emax values (i.e. maximal antidepressant-like response) and Bmax values for [^{3}H]citalopram binding in dorsal and median raphe nucleus as a function of age and SERT-genotype. In both SERT+/+ and SERT+/- mice this negative relation can be adequately described by a straight line in both the median and dorsal raphe (r ≥ 0.83-0.99, Pearson's correlation) (Figure V.E-F).

DISCUSSION

To our knowledge, we are the first to investigate the dose-dependency of escitalopram's antidepressant-like effect and its relation to SERT expression and affinity in juvenile and adolescent SERT+/+, SERT+/- and SERT-/- mice. Emax for escitalopram to reduce immobility time in the TST was lower in juvenile (P21) and adolescent (P28) mice than in adults (P90) (Figure I.F), while the potency of escitalopram was greater in younger mice than in adults (Figure I.E). These age-related variations in Emax values and potency were apparent in SERT+/+ and SERT+/- mice. SERT expression, quantified by [^{3}H]citalopram binding using autoradiography, increased with age in CA1 and CA2 regions of hippocampus, dentate gyrus and amygdala (Figure IV.A-D, Table I), and decreased with age in dorsal and median raphe nucleus (Figure IV.E-F). Age-related increases in Emax for escitalopram to reduce immobility time in the TST were positively related with age-related increases in Bmax for [^{3}H]citalopram binding (SERT expression) in hippocampus (Figure V. A-D), and negatively related with SERT expression in raphe nuclei (Figure V.F-E). Our data further support the feasibility of using juvenile and adolescent mice to study antidepressant activity (Mitchell et al., 2013), and suggest a link between antidepressant-like activity of escitalopram and relative expression of its target, SERT.

Adolescent (P28-P35) mice respond to SSRIs in the TST and FST (Bourin et al., 1998; Mason et al., 2009) and juveniles (P21) to SSRIs in the TST; however, the magnitude of effect in P21 mice was less than in adult (P90) mice (Mitchell et al., 2013). Our data in SERT+/+ mice are consistent with these findings (Figure I.B), as well as with experiments in rats that have shown escitalopram to reduce immobility in the FST in

juveniles (P21) and adolescents (P28) (Reed et al., 2008). Results from these studies parallel clinical literature suggesting SSRIs yield limited therapeutic effects in many children (Tsapakis et al., 2008; Hetrick et al., 2009; 2010).

Another population that is reported to show a limited therapeutic response to SSRIs comprise individuals carrying low expressing SERT gene variants (Serretti et al., 2007). SERT+/- mice provide a model to study these gene variants (Fox et al., 2007; Homberg and Lesch, 2011). Escitalopram reduced immobility in P21, P28 and P90 SERT+/+ and SERT+/- mice (Figure I.C), a finding consistent with previous work in adults showing that imipramine, a SERT and norepinephrine transporter blocker, and fluoxetine, an SSRI, reduced immobility time in the TST equally in SERT+/+ and SERT+/- mice (Holmes et al., 2002). SERT-/- mice did not respond to escitalopram at 10 mg/kg, a dose that is maximally effective in wild-type mice (Figure I.D), underscoring the selective nature of escitalopram's actions at SERT.

The ED_{50} for escitalopram to reduce immobility was lower in P21 and P28 SERT+/+ and SERT+/- mice than in genotype-matched P90 mice (Figure I.E). This observation is consistent with the finding that adolescent mice (P35) were more sensitive to the anti-immobility effects of low SSRI doses than adults in the FST (David et al., 2001). Pharmacokinetic factors could explain differences in antidepressant drug potency (Bylund and Reed, 2007); however, the elimination rate of escitalopram has yet to be determined in young mice. In contrast with the effects of age on the potency of escitalopram, Emax values were lower in P21 SERT+/+ and SERT+/- mice than in SERT-genotype-matched P90 mice (Figure I.F), which is consistent with and expands upon our previous studies in young SERT+/+ mice (Mitchell et al., 2013).

During postnatal development, 5-HT serves as a mitogen to regulate neuronal growth, dendritic pruning and additional aspects of neurodevelopment (Migliarini et al., 2013). Expression of SERT, as a function of age, may vary by brain region to regulate extracellular 5-HT and direct, for example, neuronal growth, which is known to vary by brain region (Daval, et al., 1987; Krogsrud et al., 2014; Sussman et al., 2016). However, there is a paucity of studies investigating antidepressant mechanisms in juvenile and adolescent mice. In an effort to account for age-related changes in antidepressant response, we quantified SERT expression using autoradiography (Figure II, III). SERT density increased with age in many serotonergic terminal regions, including the CA1 and CA2 regions of hippocampus, dentate gyrus and amygdala, but did not vary with age in the CA3 region of hippocampus, cortex or hypothalamus (Figure IV, Table I). These data partially diverge from our previous study where we used [^{3}H]citalopram saturation binding in hippocampal membrane preparations and found SERT expression in P21 mice to be the same as that in adults (Mitchell et al., 2013). This is likely because analysis of whole hippocampal homogenates does not afford the anatomical resolution of quantitative autoradiography where hippocampal sub-regions can be readily discerned. The affinity values for [^{3}H]citalopram binding in the current study (K_d: 0.54 - 1.4 nM) were similar to those in the previous study (K_d: 1.3 - 2.3 nM) (Mitchell et al., 2013).

Experiments describing SERT ontogeny in terminal regions of rat brain report peak SERT expression to occur during adolescence (~ P35) with expression decreasing to plateau in adulthood (P70-P100) (Galineau et al., 2004; Slotkin et al., 2008; Daws and Gould, 2011). In contrast, we found either no difference in SERT expression between

adolescent (P28) and adult (P90) mice (CA1, CA3, amygdala, hypothalamus and prefrontal cortex), or lower SERT expression in adolescent mice compared with adults (CA2, dentate gyrus and parietal cortex) (Figure IV, Table I). The brain regions studied possibly explain this disparity; for example, no study in rats has compared hippocampal expression of SERT across these age groups. In rats, SERT density in serotonergic cell bodies decreased from the early postnatal period to adulthood (Galineau et al., 2004; Moll et al., 2000). Our findings in dorsal and median raphe of SERT+/+ mice (Figure III, IV.E, F) are consistent with those in rats. One study compared mouse SERT mRNA levels in dorsal raphe and found they peaked at P14 and remained fairly constant from P17 – P28 (Sidor et al., 2010). Our data show a trend toward decreased [^{3}H]citalopram binding in raphe nuclei from P21 to P28; however, it is difficult to compare these data as mRNA levels do not always correlate with protein levels.

In adult mice, SERT expression has been quantified using autoradiography in brain (Bengel et al*.,* 1998; Sora et al., 2001; Montañez et al., 2003; Li et al., 2004; Perez et al., 2006). In general, these studies show that Bmax values in SERT+/- mice are ~ 50% of those in SERT+/+ mice, and SERT-/- mice show no binding beyond background levels. Our results are consistent with these reports. SERT expression in P21 and P28 SERT+/- mice revealed age-dependent changes that were similar to those in SERT+/+ mice, however, the magnitude of the changes was often smaller in SERT+/- mice (Figure IV). Differences in Bmax between P21 and P90 were only observed in CA1, amygdala and median raphe of SERT+/- mice, whereas in SERT+/+ mice, age-dependent differences were found in CA1, CA2, dentate gyrus, dorsal and median raphe (Figure IV). Because these regions are believed to be important for

antidepressant action, these results may help to explain age-related changes in antidepressant response.

Increasing extracellular 5-HT in hippocampus produces a number of actions that are thought to be necessary for the therapeutic effects of SSRIs (Campbell and McQueen, 2004; Dale et al*.,* 2016). Consistent with this, infusion of imipramine into the CA1 region of hippocampus in rats produces antidepressant-like effects in the FST (Przegaliński et al., 1997). Our data illustrate a positive relation between the Emax for escitalopram to produce antidepressant-like effects and Bmax for [^{3}H]citalopram binding in hippocampus (Figure V.A-D). Expression does not always indicate function; however, it is tempting to speculate that a developmental lag in hippocampal SERT expression could limit the antidepressant-like response to SSRIs.

In contrast, there was a negative relation between Emax for antidepressant-like response and Bmax for [^{3}H]citalopram binding in raphe nuclei (Figure V.E-F). Greater SERT expression in the raphe of juvenile and adolescent mice than in adult mice could produce age-dependent differences in brain region-specific extracellular 5-HT concentrations after SSRI administration; however, it is unclear exactly how region-specific differences in 5-HT may limit the therapeutic effects of SSRIs. Of course, age-dependent shifts in expression and/or function of 5-HT receptors may also contribute to the limited antidepressant-like response of escitalopram in young mice and should not be overlooked (Carr and Lucki, 2010). Future studies are necessary to better understand the mechanism(s) contributing to the limited antidepressant-like response of escitalopram in young mice. Moreover, because SSRIs differ in their affinity for SERT as well as other targets (including receptors and transporters), studies examining a

broader range of SSRIs may provide valuable insight into identifying antidepressants that may be more effective for the treatment of depression in juveniles.

To our knowledge, this is the first study to evaluate the ontogeny of SERT expression and antidepressant-like response in P21, P28 and P90 SERT+/+ and SERT+/- mice. Its findings help build the foundation needed to discern mechanisms underlying the limited therapeutic benefit of SSRIs in juveniles and adolescents.

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AUTHORS CONTRIBUTIONS

Participated in research design: Mitchell NC, Gould GG, Koek W, and Daws LC.

Conducted experiments: Mitchell NC and Gould GG.

Performed data analysis: Mitchell NC.

Wrote or contributed to the writing of the manuscript: Mitchell NC, Gould GG, Koek W, and Daws LC.

REFRENCES

Baganz NL, Horton RE, Calderon AS, Owens WA, Munn JL, Watts LT, Koldzic-Zivanovic N, Jeske NA, Koek W, Toney GM, and Daws LC (2008) Organic cation transporter 3: Keeping the brake on extracellular serotonin in serotonin-transporter-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* **105**: 18976-18981.

Bengel D, Murphy DL, Andrews AM, Wichems CH, Feltner D, Heils A, Mössner R, Westphal H, and Lesch KP (1998) Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter-deficient mice. *Mol. Pharmacol.* **53**: 649-655.

Bourin M, Colombel MC, Redrobe JP, Nizard J, Hascoët M, and Baker GB (1998) Evaluation of efficacies of difference classes of antidepressants in the forced swimming test in mice at different ages. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **22**: 343-351.

Bujoreanu S, Benhayon D, and Szigethy E (2011). Treatment of depression in children and adolescents. *Pediatr. Ann.* **40**: 548-555.

Bylund B and Reed A (2007). Childhood and adolescent depression: why do children and adults respond differently to antidepressant drugs? *Neurochem. Int.* **51**: 246-253.

Campbell S and McQueen G (2004) The role of the hippocampus in the pathophysiology of major depression. *J. Psychiatry Neuorsci.* **29**: 417-426.

Carr CV and Lucki I (2011) The role of serotonin receptor subtypes in treating depression: a review of animal studies. *Psychopharmacology* **213**: 265-287.

Castagné V, Moser P, Roux S, and Porsolt RD (2011) Rodent models of depression: forced swim and tail suspension behavioral despair tests in rats and mice. *Curr. Protoc. Neurosci.* Chapter 8, Unit 8.10A.

Cryan JF, Mombereau C, and Vassout A (2005) The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. *Neurosci. Biobehav. Rev.* **29**: 571-625.

Dale E, Pehrson AL, Jeyarajah T, Li Y, Leiser SC, Smagin G, Olsen CK, and Sanchez C (2016) Effects of serotonin in the hippocampus: how SSRIs and multimodal antidepressant might regulate pyramidal cell function. *C.N.S. Spectr.* **8**: 1-19.

D'Amato RJ, Largent BL, Snowman AM, and Snyder SH (1987) Selective labeling of serotonin uptake sites in rat brain by [^{3}H]citalopram contrasted to labeling of multiple sites by [^{3}H]imipramine. *J. Pharmacol. Exp. Ther.* **242**: 364-371.

Daval G, Vergé D, Becerril A, Gozlan H, Spampinato U, and Hamon, M. (1987) Transient expression of 5-HT1A receptor binding sites in some areas of the rat CNS during postnatal development. *Int. J. Dev. Neurosci.* **5**: 171-180.

David DJ, Bourin M, Hascoët M, Colombel MC, Baker G, and Jolliet P (2001) Comparison of antidepressant activity in 4- and 40-week-old male mice in the forced swim test: involvement of 5-HT1A and 5-HT-1B receptors in old mice. *Psychopharmacology* **153**: 443-449.

Daws LC and Gould GG (2011) Ontogeny and regulation of the serotonin transporter: providing insight into human disorders. *Pharmacol. Ther.* **131**: 61-79.

Fox MA, Andrews AM, Wendland JR, Lesch K, Holmes A, and Murphy DL (2007) A pharmacological analysis of mice with a targeted disruption of the serotonin transporter. *Psychopharmacology* **195**: 147-166.

Galineau L, Koda E, Guilloteau D, Vilar MP, and Chalon S (2004) Ontogeny of the dopamine and serotonin transporters in the rat brain: an autoradiographic study. *Neurosci. Lett.* **363**: 266-271.

Hetrick SE, McKenzie JE, and Merry SN (2010) The use of SSRIs in children and adolescents. *Curr. Opin. Psychiatry* **23**: 53-57.

Hetrick SE, Merry SN, McKenzie J, Sindahl P, and Proctor M (2009) Selective serotonin reuptake inhibitors (SSRIs) depressive disorders in children and adolescents (Review). *Cochrane Database Syst. Rev.* 1-91.

Holmes A, Yang RJ, Murphy DL, and Crawley JN (2002) Evaluation of antidepressant-related behavioral response in mice lacking the serotonin transporter. *Neuropsychopharmacology* **27**: 914-923.

Homberg JR and Lesch K (2011) Looking on the bright side of serotonin transporter gene variation. *Biol. Psychiatry* **69**: 513-519.

Horton RE, Apple DM, Owens WA, Baganz NL, Cano S, Mitchell NC, Vitela M, Gould GG, Koek W, and Daws LC (2013) Decynium-22 enhances SSRI-induced

antidepressant-like effects in mice: uncovering novel targets to treat depression. *J. Neurosci.* **33**: 10534-10543.

Kessler RC, Avenevoli S, and Reis Merikangas K (2001). Mood disorders in children and adolescents: an epidemiologic perspective. *Biol. Psychiatry* **49**: 1002-1014.

Koek W, Mercer SL, Coop A, and France CP (2009) Behavioral effects of gamma-hydroxybutyrate, its precursor gamma-butyrolactone, and $GABA_B$ receptor agonist: time course and differential antagonism by the $GABA_B$ receptor antagonist 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP35348). *J. Pharmacol. Exp. Ther.* **330***:* 876-883.

Krogsrud SK, Tamnes CK, Fjell AM, Amlien I, Grydeland H, Sulutvedt U, Due-Tønnessen P, Bjørnerud A, Sølsnes AE, Håberg AK, Skrane J and Walhovd KB (2014) Development of hippocampal subfield volumes from 4 to 22 years. *Hum. Brain Mapp.* **35**: 5645-5657.

Li Q, Ma L, Innis RB, Seneca N, Ichise M, Huang H, Laruelle M, and Murphy DL (2004) Pharmacological and genetic characterization of two selective serotonin transporter ligands: 2-[2-(dimethylaminomethylphenylthio)]-5-fluoromethylphenylamine (AFM) and 3-amino-4-[2-(dimethulaminomethyl-phenylthio)]benzonitrile (DASB). *J. Pharmacol. Exp. Ther.* **308**: 481-486.

Loizou LA and Salt P (1970) Regional changes in monoamines of the rat brain during postnatal development. *Brain Res.* **20**: 467-470.

Mason SS, Baker KD, Davis KW, Pogorelov VM, Malbari MM, Ritter R, Wray SP, Gerhardt B, Lanthorn TH, and Savelieva KV (2009) Differential sensitivity to

SSRI and tricyclic antidepressants in juvenile and adult mice of three strains. *Eur. J. Pharmacol.* **602**: 306-315.

Migliarini S, Pacini G, Pelosi B, Lunardi G, and Pasqualetti M (2013) Lack of brain serotonin affects postnatal development and serotonergic neuronal circuitry formation. *Mol. Psychiatry* **18**: 1106-1118.

Miranda-Contreras L, Mendoza-Brieño RV, and Palacios-Prü EL (1998) Levels of monoamine and amino acid neurotransmitters in developing male mouse hypothalamus and in histotypic hypothalamic cultures. *Inter. J. Dev. Neurosci.* **16**: 403-412.

Mitchell NC, Gould GG, Smolik CM, Koek W, and Daws LC (2013) Antidepressant-like drug effects in juvenile and adolescent mice in the tail suspension test: Relationship with hippocampal serotonin and norepinephrine transporter expression and function. *Front Pharmacol.* **4**: 131.

Mitchell NC, Koek W, and Daws LC (2015) Antidepressant-like effects and basal immobility depend on age and serotonin transporter genotype. *Genes Brain Behav.* **14**: 543-549.

Moll GH, Mehnert C, Wicker M, Bock N, Rothenberger A, Rüther E, and Huether G (2000) Age-associated changes in the densities of presynaptic monoamine transporters in different regions of the rat brain from early juvenile life to late adulthood. *Brain Res. Dev. Brain Res.* **119**: 251-257.

Montañez S, Owens WA, Gould GG, Murphy DL, and Daws LC (2003) Exaggerated effect of fluvoxamine in heterozygote serotonin transporter knockout mice. *J. Neurochem.* **86**: 210-219.

O'Leary OF, Bechtholt AJ, Crowely JJ, Hill TE, Page ME, and Lucki I (2007) Depletion of serotonin and catecholamines block the acute behavioral response to different classes of antidepressant drugs in the mouse tail suspension test. *Psychopharmacology* **192**: 357-371.

Paxinos G and Franklin KBJ (1997) *The Mouse Brain in Stereotaxic Coordinates*, Academic Press, San Diego, California.

Perez XA, Bianco LE, and Andrews AM (2006) Filtration disrupts synaptosomes during radiochemical analysis of serotonin uptake: comparison with chronoamperometry in SERT knockout mice. *J. Neurosci. Methods* **30**: 245-255.

Petersen IT, Bates JE, Goodnight JA, Dodge KA, Lansford JE, Pettit GS, Latendresse SJ, and Dick DM (2012) Interaction between serotonin transporter polymorphism (5-HTTLPR) and stressful life events in adolescents' trajectories of anxious/depressed symptoms. *Dev. Psychol.* **48**: 1463-1475.

Przegaliński E, Tatarczyńska E, Dereń-Wesolek A, and Chojnacka-Wojcik E (1997) Antidepressant-like effects of a partial agonist at strychnine-insensitive glycine receptors and a competitive NMDA receptor antagonist. *Neuorpharmacology* **36**: 31-37.

Reed AL, Happe HK, Petty F, and Bylund DB (2008) Juvenile rats in the forced-swim test model the human response to antidepressant treatment for pediatric depression. *Psychopharmacology* **197**: 433-441.

Ripoll N, David DJ, Dailly E, Hascoët M, and Bourin M (2003) Antidepressant-like effects in various mice strains in the tail suspension test. *Behav. Brain Res.* **143**: 193-200.

Serretti A, Kato M, De Ronchi D, and Kinoshita T (2007) Meta-analysis of serotonin transporter gene promotor polymorphism (5HTTLPR) association with selective serotonin reuptake inhibitor efficacy in depressed patients. *Mol. Psychiatry* **12**: 247-257.

Sidor MM, Amath A, Macqueen G, and Foster JA (2010) A developmental characterization of mesolimbocortical serotonergic gene expression changes following early immune challenge. *Neuroscience* **171**: 734-746.

Slotkin TA, Ryde IT, Levin ED, and Seidler FJ (2008) Developmental neurotoxicity of low dose diazinon exposure of neonatal rats: Effects on serotonin systems in adolescence and adulthood. *Brain Res. Bull.* **75**: 640-647.

Sora I, Hall FS, Andrews AM, Itokawa M, Li XF, Wei HB, Wichems C, Lesch KP, Murphy DL, and Uhl GR (2001) Molecular mechanisms of cocaine reward: combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. *Proc. Natl. Acad. Sci. U.S.A.* **98**: 5300-5305.

Spear LP (2000) The adolescent brain and age-related behavioral manifestations. *Neurosci. Behav. Rev.* **24**: 417-463.

Steru L, Chermat R, Thierry B, and Simon P (1985) The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology* **85**: 367-370.

Sussman D, Leung RC, Chakravarty MM, Lerch JP, and Taylor MJ (2016) Developing human brain: age-related changes in cortical, subcortical, and cerebellar anatomy. *Brain Behav.* **6**: e00457.

Tsapakis EM, Soldani F, Tondo L, and Baldessarini RJ (2008). Efficacy of antidepressants in juvenile depression: meta-analysis. *Br. J. Psychiatry* **193**: 10-17.

Wallace JA and Lauder JM (1983) Development of the serotonergic system in the rat embryo: an immunocytochemical study. *Brain Res. Bull.* **10**: 459-479.

FOOTNOTES

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a) Data from this manuscript will be reprinted in a thesis (thesis title and publication date TBA).

b) Reprint request for thesis will be by Nathan Mitchell, 7703 Floyd Curl Drive, San Antonio, TX, 78229-3900, USA.

FIGURE LEGENDS

Figure I. Escitalopram reduces immobility time in the TST in juvenile, adolescent and adult mice as a function of SERT genotype. (A) Basal immobility time (saline-injected controls) in seconds across age and SERT-genotype. Solid symbols represent a significant difference from P21 with Dunnett's *post hoc* multiple comparisons test after a two-factor ANOVA (age, SERT-genotype) * $P < 0.05$ represent difference from age matched SERT+/+ and # $P < 0.05$ represent difference from age matched SERT+/- with Tukey's *post hoc* multiple comparisons test. (B-D) Escitalopram dose-effect curves across age in SERT+/+, SERT+/- and SERT-/- mice. Immobility expressed as a percent of saline-injected controls. Solid symbols represent a significant difference from vehicle treatment with Dunnett's *post hoc* multiple comparisons test after a two-factor ANOVA (age, drug dose) performed separately for each genotype. * $P < 0.05$, ** $P < 0.01$ represent significant difference from SERT-gene matched P90; Tukey's *post hoc* multiple comparisons test. (E) Half maximally effective dose (ED_{50}); * $P < 0.05$, ** $P < 0.01$ represent significant difference from SERT-gene matched P90; † $P < 0.05$ P90 SERT+/+ vs P90 SERT+/-. (F) Maximal effect (Emax, expressed as a percent from control) of dose-effect curves B and C; * $P < 0.05$, ** $P < 0.01$ represent significant difference from SERT-gene matched P90 with Tukey's *post hoc* multiple comparisons test after a two-factor ANOVA (age, SERT-genotype). There were no statistically significant main effects of sex or sex x dose interactions in any age group or SERT-genotype, so data for both genders were pooled. Data are mean ± SEM, except for ED_{50} values, which are expressed as mean. SERT+/+ n = 17 – 31 (males n = 4 - 16 and females n = 9 – 21 pooled), SERT+/- n = 14 – 20 (males n = 8 - 10 and females n = 6 - 11 pooled), and SERT-/- n = 18 – 26 (males n = 9 - 14 and females 8 – 12 pooled), per data point.

Figure II. Specific binding of [^{3}H]citalopram to SERT in hippocampal subregions as a function of age and SERT genotype. Brain sections from P21 (▲), P28 (■) and P90 (●) mice

were incubated with increasing concentrations of [^{3}H]citalopram. Non-specific binding was defined by sertraline (20µM). (A) Representative coronal sections showing [^{3}H]citalopram binding at the level of plate 47 (Paxinos and Franklin, 1997) in P21, P28 and P90, SERT+/+, SERT+/- and SERT-/- mice. Saturation binding isotherms in CA1 (B), CA2 (C), CA3 (D) regions of hippocampus and dentate gyrus (E) of P21, P28 and P90, SERT+/+, SERT+/- and SERT-/- mice. Bmax values for each curve are summarized in Figure IV. There were no significant differences in K_d among ages or between SERT+/+ and SERT+/- mice. There was no significant difference between males and females for any age or SERT-genotype, so data for both genders are pooled SERT+/+ n = 8 – 11 (males n = 5 and females n = 3 – 6 pooled), SERT+/- n = 5 – 10 (males n = 3 – 5 and females 2 – 5 pooled) and SERT-/- n = 2 – 4 (males n = 1 – 2 and females n = 1 – 2 pooled), mice per group. Note that because binding in SERT-/- mice was not different from background the sample size was not further increased.

Figure III. Specific binding of [^{3}H]citalopram to SERT in dorsal and median raphe nuclei as a function of age and SERT genotype. Brain sections from P21 (▲), P28 (■) and P90 (●) mice were incubated with increasing concentrations of [^{3}H]citalopram. Non-specific binding was defined by sertraline (20µM). (A) Representative coronal sections showing [^{3}H]citalopram binding at the level of plate 64 (Paxinos and Franklin, 1994) in P21, P28 and P90, SERT+/+, SERT+/- and SERT-/- mice. Saturation binding isotherms in dorsal raphe (B) and median raphe (C) of P21, P28 and P90, SERT+/+, SERT+/- and SERT-/- mice. Bmax values for each curve are summarized in Figure IV. There were no significant differences in K_d among ages or between SERT+/+ and SERT+/- mice. There was no significant difference between males and females for any age or SERT-genotype, so data for both genders are pooled. SERT+/+ n = 8 – 11 (males n = 5 and females n = 3 – 6 pooled), SERT+/- n = 5 – 10 (males n = 3 – 5 and females 2 – 5 pooled) and SERT-/- n = 2 – 4 (males n = 1 – 2 and females n = 1 – 2 pooled),

mice per group. Note that because binding in SERT-/- mice was not different from background the sample size was not further increased.

Figure IV. Bmax for [^{3}H]citalopram binding to SERT in SERT+/+ and SERT+/- mice aged P21, P28 and P90. Bmax values were determined from 1-site curve fits to data plotted in Figure II.B-D and Figure III.B-C from P21 (red), P28 (blue) and P90 (black) SERT +/+ and SERT+/- mice. Data are mean ± SEM pooled from male and female mice. * $P < 0.05$, ** $P < 0.01$ represent significant difference from SERT-gene matched P90, Tukey's *post hoc* multiple comparisons test after a repeated measure two-factor ANOVA (age, brain region). SERT+/+ n = 8 – 11 (males n = 5 and females n = 3 – 6 pooled) and SERT+/- n = 5 – 10 (males n = 3 – 5 and females 2 – 5 pooled).

Figure V. Relationship between Emax values for escitalopram to reduce immobility time in the TST and Bmax values for specific [^{3}H]citalopram binding in hippocampal and raphe subregions as a function of age and SERT genotype. P21 (▲), P28 (■) and P90 (●) mice. The CA1 **(A)**, CA2 **(B)**, CA3 **(C)** regions of hippocampus, dentate gyrus **(D)**, dorsal raphe **(E)** and median raphe **(F)** are shown. Data taken from Figure I (SERT+/+ n = 18 – 20 and SERT+/- n = 16 - 19) and Figure IV (SERT+/+ n = 8 – 11 and SERT+/- n = 5 – 10). Data are mean ± SEM, male and female data pooled.

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TABLES

Table I. Summary of Bmax values for specific [^{3}H]citalopram binding in SERT+/+ and SERT+/- mice.

Genotype	P21	P28	P90
Prefrontal cortex			
SERT+/+	568 ± 25	588 ± 25	583 ± 18
SERT+/-	322 ± 32	331 ± 15	363 ± 28
Parietal cortex			
SERT+/+	342 ± 24*	382 ± 21	431 ± 24
SERT+/-	186 ± 37	214 ± 24	225 ± 22
Amygdala			
SERT+/+	970 ± 31	1032 ± 29	1058 ± 29
SERT+/-	669 ± 58	678 ± 21*	801 ± 48
Ventromedial hypothalamus			
SERT+/+	999 ± 38	933 ± 37	938 ± 30
SERT+/-	696 ± 57	677 ± 29	597 ± 37

Data are mean ± SEM fmol/mgpr. There was no significant difference between males and females for any age or SERT-genotype, so data for both genders are pooled. * $P < 0.05$ different from SERT genotype-matched P90 group; Tukey's multiple comparisons test after two-factor ANOVA, n = 8 – 11.

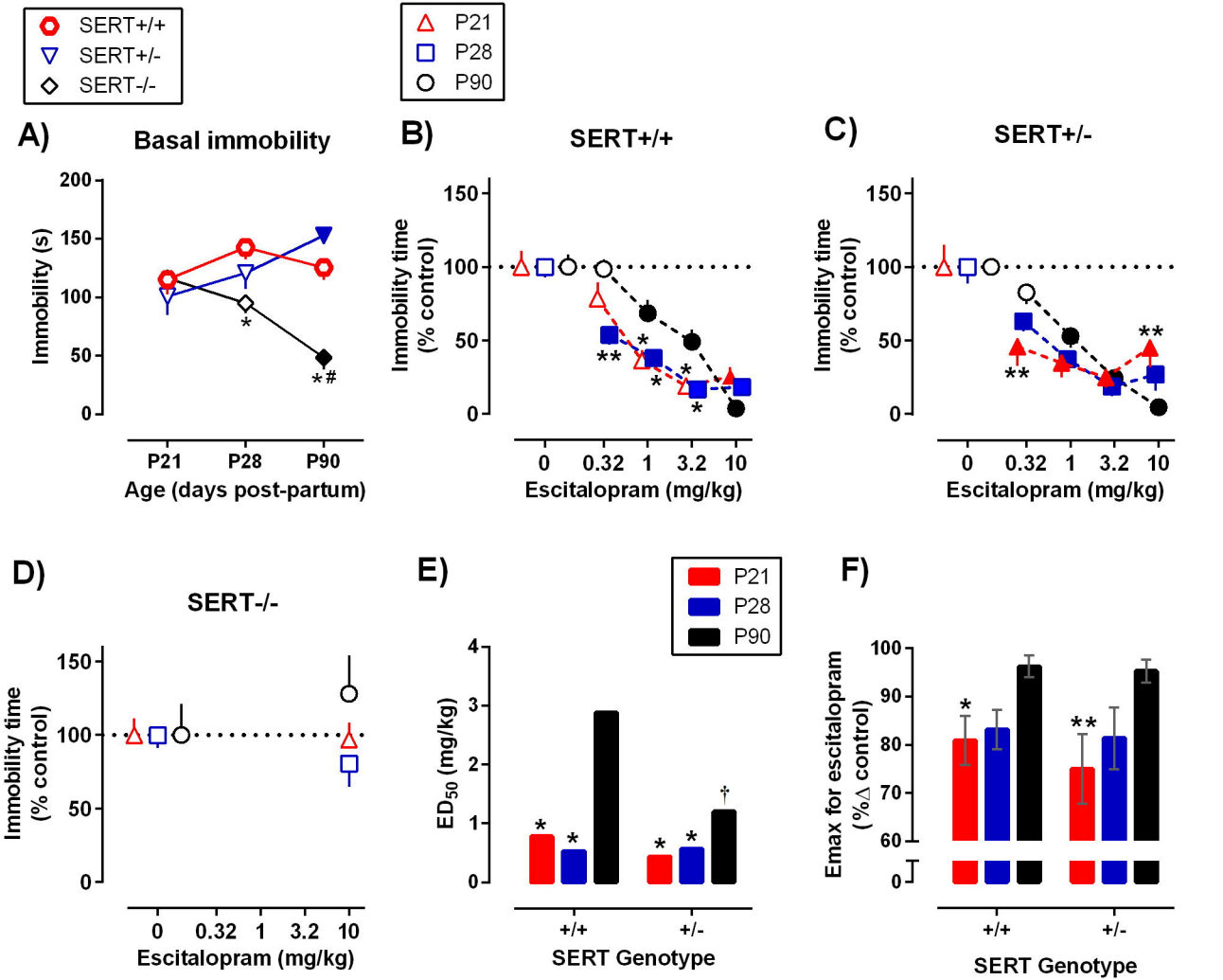
SERT+/+
SERT+/-
SERT-/-
P21
P28
P90
A)
Basal immobility
Immobility (s)
200
150
100
50
0
P21
P28
P90
Age (days post-partum)
B)
SERT+/+
Immobility time (% control)
150
100
50
0
0
0.32
1
3.2
10
Escitalopram (mg/kg)
C)
SERT+/-
Immobility time (% control)
150
100
50
0
0
0.32
1
3.2
10
Escitalopram (mg/kg)
D)
SERT-/-
Immobility time (% control)
150
100
50
0
0
0.32
1
3.2
10
Escitalopram (mg/kg)
E)
P21
P28
P90
ED50 (mg/kg)
4
3
2
1
0
+/+
+/-
SERT Genotype
F)
Emax for escitalopram (%Δ control)
100
90
80
70
60
0
+/+
+/-
SERT Genotype

Figure 1.

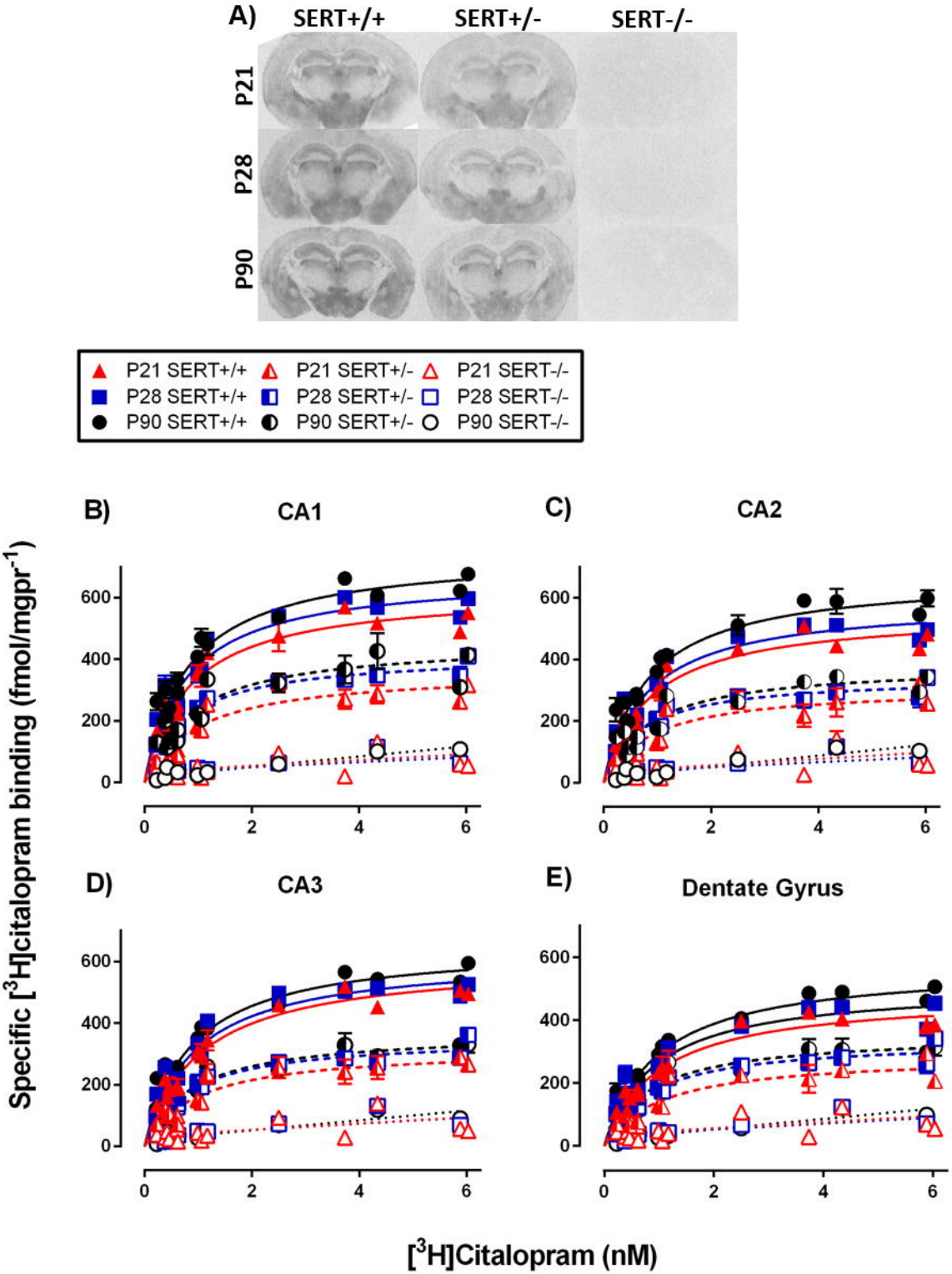
A)
SERT+/+
SERT+/-
SERT-/-
P21
P28
P90
P21 SERT+/+
P21 SERT+/-
P21 SERT-/-
P28 SERT+/+
P28 SERT+/-
P28 SERT-/-
P90 SERT+/+
P90 SERT+/-
P90 SERT-/-
B)
CA1
C)
CA2
D)
CA3
E)
Dentate Gyrus
Specific [3H]citalopram binding (fmol/mgpr-1)
[3H]Citalopram (nM)

Figure 2.

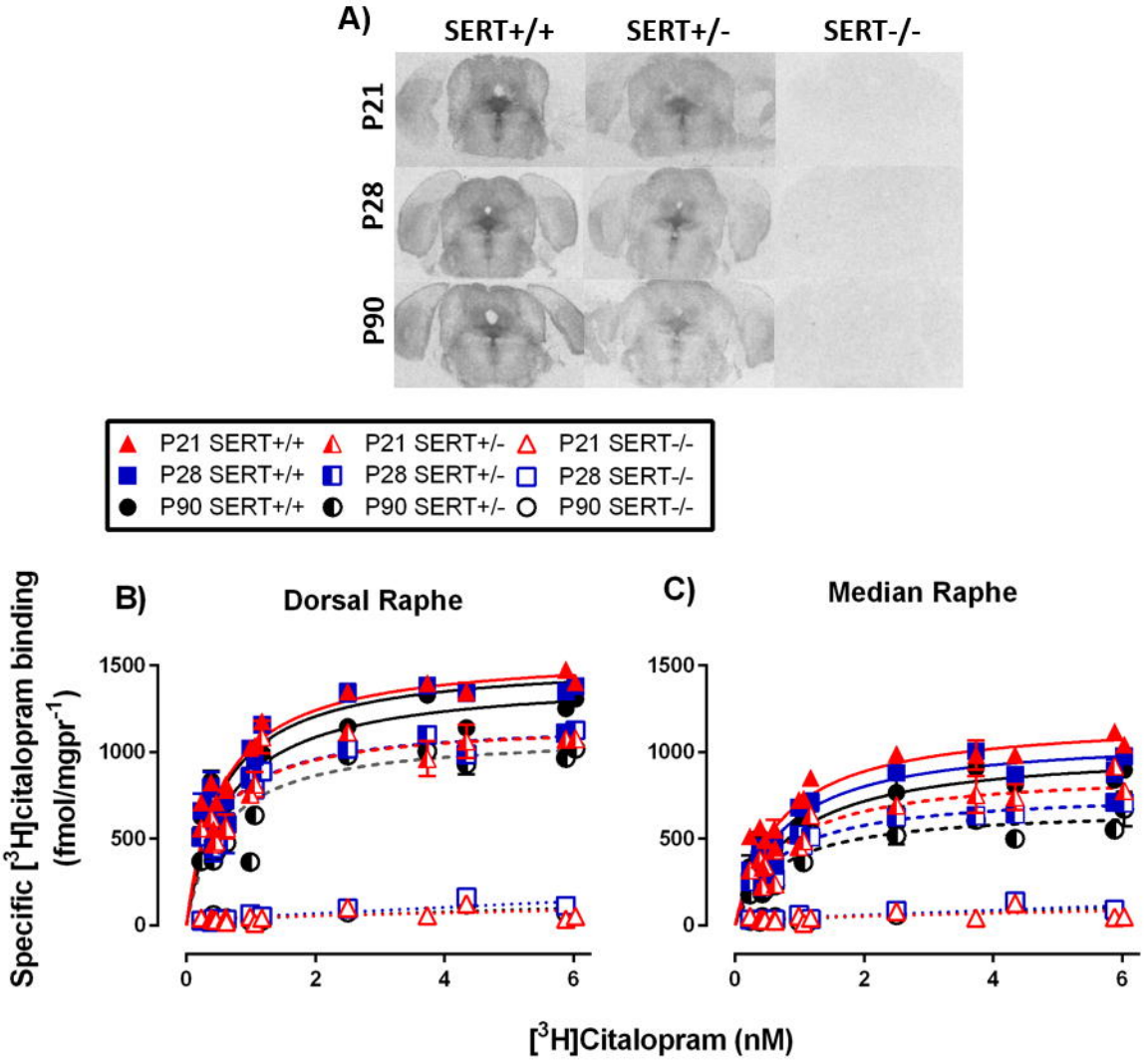
A)
SERT+/+
SERT+/-
SERT-/-
P21
P28
P90
P21 SERT+/+
P21 SERT+/-
P21 SERT-/-
P28 SERT+/+
P28 SERT+/-
P28 SERT-/-
P90 SERT+/+
P90 SERT+/-
P90 SERT-/-
B)
Dorsal Raphe
C)
Median Raphe
Specific [3H]citalopram binding (fmol/mgpr-1)
1500
1000
500
0
0
2
4
6
[3H]Citalopram (nM)

Figure 3.

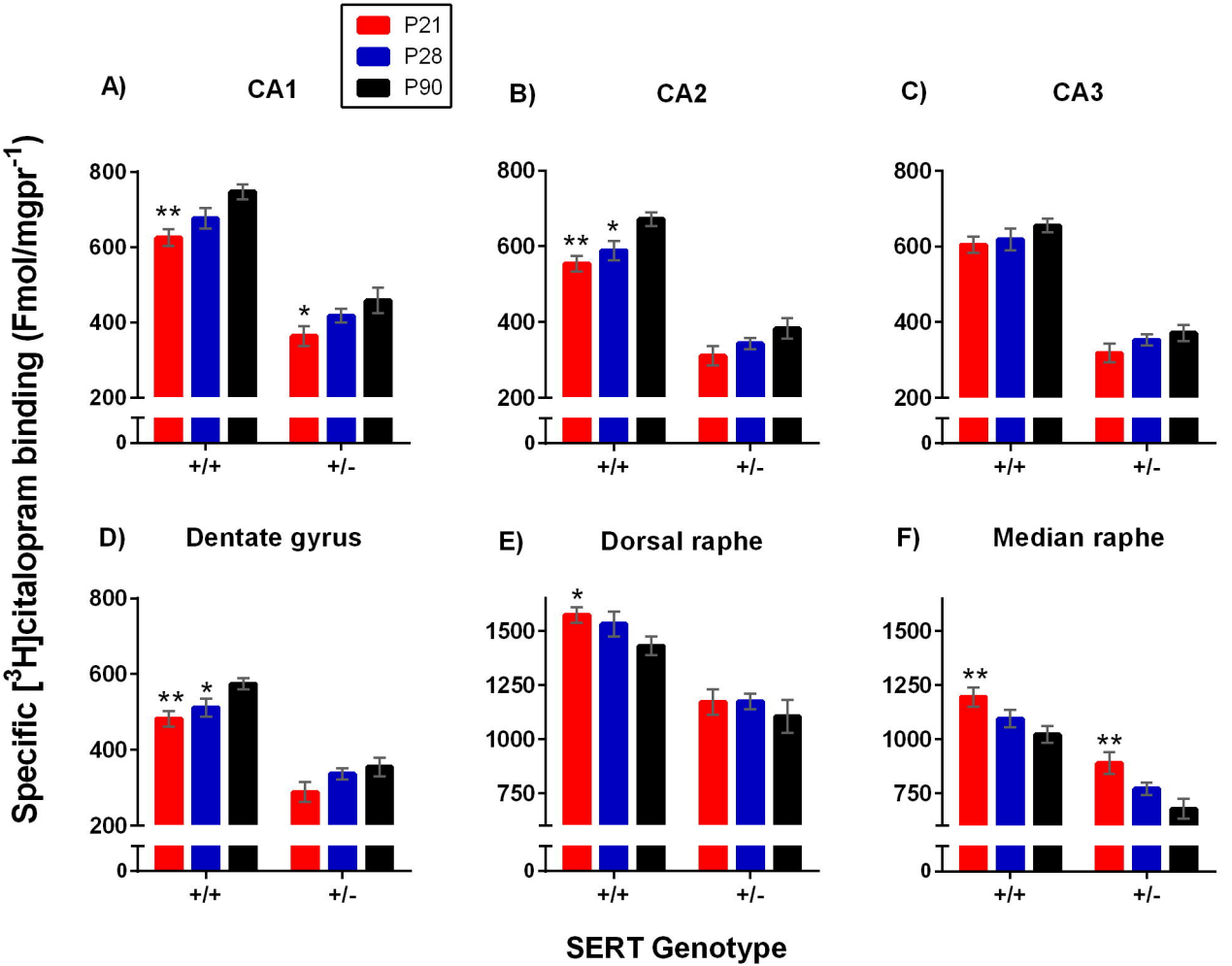
P21
P28
P90
A)
CA1
B)
CA2
C)
CA3
D)
Dentate gyrus
E)
Dorsal raphe
F)
Median raphe
Specific [3H]citalopram binding (Fmol/mgpr-1)
SERT Genotype
+/+
+/-

Figure 4.

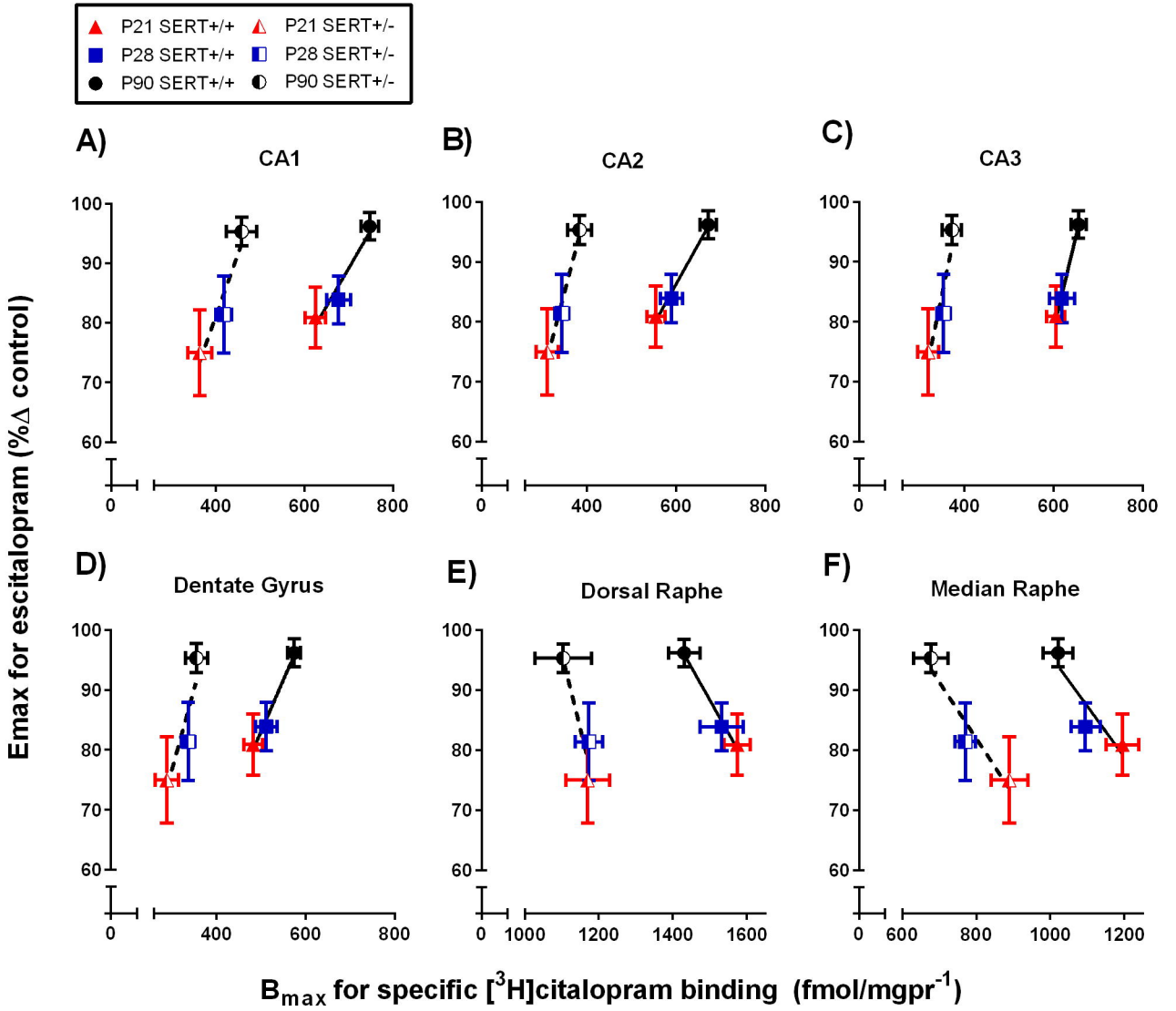
P21 SERT+/+
P21 SERT+/-
P28 SERT+/+
P28 SERT+/-
P90 SERT+/+
P90 SERT+/-
A)
CA1
B)
CA2
C)
CA3
D)
Dentate Gyrus
E)
Dorsal Raphe
F)
Median Raphe
Emax for escitalopram (%Δ control)
B_{max} for specific [^{3}H]citalopram binding (fmol/mgpr^{-1})

Figure 5.